

Nitrogen-status sensing in Arabidopsis

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Abstract

Plant nitrogen use efficiency (NUE) is important in agriculture because it significantly influences yield and fertiliser requirements. Many factors influence NUE; these include nitrogen availability, internal nitrogen-status, nitrogen uptake and assimilation rates as well as carbon status. Previous attempts to improve NUE by targeting the main nitrogen uptake or assimilation machinery (e.g. over-expression of nitrate reductase or glutamine synthetase) have had mixed results. This is because these enzymes are strictly controlled by multiple feedback mechanisms relating to internal and external nitrogen-status. Arguably, a better approach to manipulating NUE would be to identify and suppress these feedback signals either alone, or in conjunction with over-expression of the uptake and assimilation machinery. This could enhance exploitation of nitrogen resources leading to increased growth.

Internal nitrogen-status signalling is poorly understood. However, it is known that amino acids, when exogenously supplied, inhibit the uptake of nitrate. The mechanism for this inhibition has not been identified, but it is likely the result of sensing of internal nitrogen status, the amino acids representing a signal of nitrogen sufficiency, in turn leading to reduced expression and activity of the nitrogen uptake and assimilation machinery. In this thesis I conducted a reverse-genetic screen based on the response of candidate amino acid transporter knock-out mutants to exogenous alanine. In wild-type *Arabidopsis*, exogenous alanine caused inhibition of vegetative growth, which is accompanied by a reduction in nitrate uptake and transcripts encoding nitrate transporters. In the reverse-genetic growth screen, I identified four mutants with vegetative growth that was insensitive to exogenous alanine. Further characterisation of these mutants revealed that nitrogen uptake was not inhibited and this was accompanied by a lack of repression of nitrate transporter transcripts. Based on this evidence, I concluded that these amino acid transporter mutants were perturbed in nitrogen-status signalling. However, the origin of this signalling perturbation remains unclear. I formulated two hypotheses to explain how four amino acid transporter mutants could have the same signalling phenotype. The first suggested that the four alanine-insensitive transporters could form a functional complex, which is part of the nitrogen-status signalling pathway. The second suggested that the loss-of-function of the amino acid transporter perturbs metabolite levels, in a common way in each mutant, producing a metabolic signature which is sensed downstream of the transporter by an unknown mechanism. The latter hypothesis was tested by extensive metabolite profiling and I could find no evidence in support of the hypothesis. Preliminary investigations were made of the functional-complex hypothesis but technical problems prevented significant progress.

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Abbreviations

AAP	Amino acid permease
AE	Agronomic efficiency
AIB	Alpha-aminoisobutyric acid
CAT	Cationic amino acid transporter
CBP	Calmodulin-binding protein
cDNA	Complementary DNA
CTAG	C-terminal TAP-tag
DAS	Days after sowing
DEPC	Diethylpyrocarbonate
DON	6-diazo-5-oxo-L-norleucine
ECL	Enhanced chemiluminescent
GC-MS	Gas chromatography – mass spectrometry
gDNA	Genomic DNA
GFP	Green fluorescence protein
GOGAT	Glutamate oxoglutarate aminotransferase
GS	Glutamine synthetase
HRP	Horse radish peroxidase
IMAC	Immobilised metal ion affinity chromatography
LC-MS	Liquid chromatography – mass spectrometry
LHT	Lysine histidine transporter
MS	Murashige and Skoog
MSFTA	<i>N</i> -methyl- <i>N</i> -(trimethylsilyl)trifluoroacetamide)
MSX	Methionine sulfoxamine
MVA	Mevalonate pathway
NRT	Nitrate transporter
NTAG	N-terminal TAP-tag
NUE	Nitrogen-use efficiency

NUpE	Nitrogen uptake efficiency
NUtE	Nitrogen utilisation efficiency
PAGE	Polyacrylamide gel electrophoresis
PAP	Peroxodase anti-peroxidase
PNR	Primary nitrate response
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAM	Significance of microarray
SEC	Size exclusion chromatography
SEM	Standard error of the mean
TAP	Tandem affinity purification
tBDMS	tert-butyldimethylsilyl ether
TBS	Tris buffered saline
T-DNA	transfer-DNA
TEV	Tobacco etch virus
UTR	Untranslated region

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Chapter 1: General Introduction

1.1 Thesis aims

Given the importance of nitrogen-status signalling for the plant and the potential for it to be manipulated for the improvement of nitrogen-use efficiency (NUE), it is vital that the molecular details of the mechanisms of nitrogen-status signalling are uncovered. Although it is clear that concentrations of various amino acids are sensed by the plant for this purpose, the sensing and signalling mechanisms have not been elucidated. In an attempt to elucidate these mechanisms, this thesis will focus on identifying amino acid transporters that function as transceptors to sense amino acid availability.

Previous attempts to improve NUE by targeting nitrogen uptake or assimilation machinery have had mixed results. For example, tobacco plants overexpressing rice chloroplastic *GS2* displayed no increase in biomass (Kozaki and Takeba, 1996), whereas *Lotus corniculatus* plants overexpressing *Glycine max* (soybean) *GS* (Vincent et al., 1997), and tobacco overexpressing *Pisum sativum* (pea) cytosolic *GS1* (Oliveira et al., 2002), did show an increase in biomass. The inconsistencies seen when overexpressing this enzyme could be due to the fact that it is strictly controlled by multiple feedback mechanisms relating to internal and external nitrogen-status signalling. Overexpressing genes encoding NR also did not increase yield or growth in various plant species, again probably due to the strict regulation of this enzyme (Curtis et al., 1999; Djennane et al., 2002a; Djennane et al., 2002b). Arguably a better approach to manipulating NUE would be to target the mechanisms that feedback internal nitrogen-status to regulate nitrogen uptake and assimilation. Suppression of these feedback mechanisms, either alone or in conjunction with overexpression of the uptake and assimilation machinery could allow superior exploitation of nitrogen resources and this may lead to increased growth. This thesis aims to uncover some of the mechanisms for nitrogen-status sensing by using reverse genetics to screen candidate amino acid knockout mutant lines for perturbed nitrogen-status signalling. Amino

acid transporters have been selected as candidates to screen based on evidence in animals of amino acid transceptors. Amino acid transceptors are transporters with sensory functions and examples include GAP1 in yeast, PATH in *Drosophila* and SLC38A9 in humans which will be discussed in detail later in this introduction. Given the existence of these transceptors in animals, it is highly likely that transceptors may also function in plants. This coupled with the knowledge that some amino acids, when exogenously supplied inhibit nitrate uptake in plant roots. The mechanism for this inhibition is currently poorly understood, but it is likely a result of sensing of internal nitrogen-status, the amino acids representing a signal of nitrogen sufficiency, in turn leading to reduced expression or activity of the nitrogen uptake and assimilation machinery.

1.2 Definition of and factors affecting nitrogen-use efficiency

Nitrogen is a major macronutrient which is essential for plant growth and development. Within the plant, nitrogen is incorporated into important molecules such as amino acids, nucleic acids and chlorophyll. The efficiency with which plants utilise nitrogen is important in agriculture because this will significantly influence yield as well as the amount of fertiliser needed. A range of factors can influence nitrogen use efficiency (NUE) and these include nitrogen availability, internal nitrogen status, N uptake and assimilation rate, carbon status, as well as genotypic differences (Xu et al., 2012). Various definitions of NUE have been proposed, which differ depending on the type of plant being analysed and the measure of nitrogen use (Good et al., 2004). For example, NUE can be determined in terms of incorporation of nitrogen into total plant biomass (Siddiqi and Glass, 1981) or solely into harvested organs of crops such as seeds (Moll et al., 1982). For most crop plants, NUE is defined as the grain yield per unit of nitrogen available from the soil (Masclaux-Daubresse et al., 2010). NUE can also be further broken down into its two main contributing factors, nitrogen uptake efficiency (NUpE), and nitrogen utilisation (or assimilation) efficiency (NUE).

NUtE is dependent on the apparent nitrogen recovery (AR) rate, which is a measure of the efficiency of the capture of nitrogen from the soil. NUtE is related to agronomic efficiency (AE) which is a measure of the efficiency of converting nitrogen to grain yield (Good et al., 2004). The multiple definitions available and the number of measurable components that make up NUE illustrate its complex nature.

1.3 The importance of improving nitrogen-use efficiency

Nitrogen is an abundant element; in its gaseous form N_2 comprises around 80% of the Earth's atmosphere, yet plants are unable to access this vast supply. They do not possess the enzymes necessary to fix gaseous N_2 . Instead plants are dependent upon the presence of dissolved inorganic N-containing ions such as NO_3^- , NO_2^- and NH_4^+ in soil. For intensive production of annual crops soil nitrogen content needs to be maintained by application of nitrogen-rich fertilisers. Indeed, farmers currently add around 85 million metric tons (MMt) of nitrogenous fertilisers to soil globally, annually, compared to only 1.3 MMt in 1930 (Frink et al., 1999). With a growing population and therefore a demand for even higher yields of crops, the amount of fertiliser use is predicted to increase further to 240 MMt by the year 2050 (Tilman, 1999). Of the additional nitrogen added to the soil, it is estimated that between 50-70% is lost through leaching due to the solubility of NO_3^- (Pilbeam, 1996). The leaching of NO_3^- from the soil causes increased soil acidification (Guo et al., 2010) which can be associated with a reduction in yield in some crops such as barley and wheat (Zhao et al., 2010), and eutrophication of surrounding bodies of water, resulting in the death of aquatic organisms (U.S. Environmental Protection Agency. 2007). In addition to the direct environmental costs, the production of NH_3 for fertilisers by the Haber Bosch process is energy intensive and consumes substantial amounts of fossil fuel contributing to global greenhouse gas emissions and climate change. For all these reasons it is becoming increasingly more important to develop plants which are more nitrogen-use efficient; in order

to protect the environment, reduce agricultural costs and feed the population, nitrogen fertiliser input must be reduced without resulting in yield losses.

1.4 The process of nitrogen assimilation in plants

Transporters in the plasma membrane of root cells facilitate the specific uptake of NO_3^- , NO_2^- and NH_4^+ from the soil. Once inside the cell, nitrate can be transported to the vacuole for storage, reduced to nitrite in a reaction catalysed by the enzyme nitrate reductase in the cytosol of the root (Figure 1.1), or translocated to the shoot to be reduced to nitrite. Nitrate assimilation in higher plants largely occurs in the shoots rather than roots due to the requirement of a reducing power which is readily available in photosynthetic tissues (Andrews, 1986). In *Arabidopsis*, shoot translocation is mediated by the nitrate transporter NRT1.5 which enables the loading and unloading of nitrate into the xylem (Lin et al., 2008). In the leaf, nitrite is then further reduced to ammonium by nitrite reductase in the plastid. Ammonium is then assimilated into glutamine, catalysed by glutamine synthetase (GS). The pool of glutamine generated in this reaction can then be used to donate the N from its amine group to form glutamate via a transamination reaction catalysed by the enzyme glutamate oxoglutarate aminotransferase (GOGAT). This reaction requires a carbon skeleton in the form of 2-oxoglutarate from the tricarboxylic acid (TCA) cycle to produce two molecules of glutamate. The glutamate produced is used in further aminotransferase reactions to produce various other amino acids, or can be cycled back to the GS reaction to produce glutamine. This GS/GOGAT cycle (Figure 1.1) ensures that adequate pools of glutamate and glutamine, the major nitrogen donor amino acids, are maintained (Lam et al., 1996).

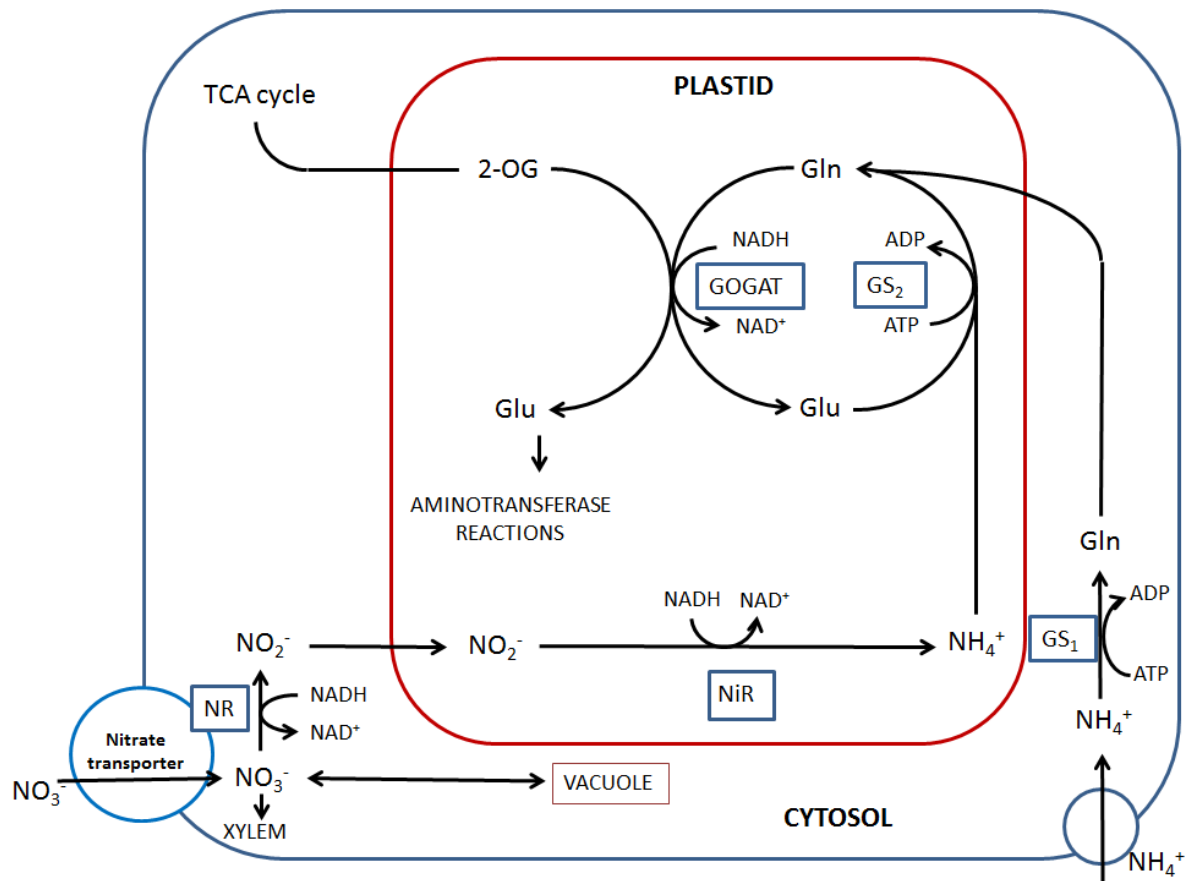


Figure 1.1: Nitrogen uptake and assimilation.

Diagram of nitrate uptake and its subsequent assimilation into amino acids taking place in the cytosol and the plastid of a root cell. Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; GS₁, glutamine synthetase1; GS₂, glutamine synthetase2; Gln, glutamine; GOGAT, glutamate oxoglutarate aminotransferase; Glu, glutamate; 2-OG, 2-oxoglutarate and TCA cycle, tricarboxylic acid cycle.

1.5 Nitrate as a signal molecule

1.5.1 Nitrate sensing triggers the Primary Nitrate Response

Plants have the capacity to sense the availability of nitrogen in the soil and the internal nitrogen status of their cells. This enables them to coordinate their development, physiology and metabolism with the amount of nitrogen available in order to maximise their chances of survival in soil where nitrate concentrations are variable. To date there is evidence for the existence of a variety of sensing and signalling mechanisms in plants, the best characterised of which is the primary nitrate response (PNR) (Redinbaugh and Campbell, 1991; Gowri et al., 1992). The events occurring immediately after nitrate provision constitute the PNR. The response does not require *de novo* protein synthesis and involves a rapid increase in levels of transcripts encoding nitrate assimilatory enzymes such as nitrate reductase, and nitrate transporters such as Arabidopsis NRT1.1 and NRT2.1 (Gowri et al., 1992; Crawford, 1995; Stitt, 1999; Wang et al., 2000; Wang et al., 2003). This response still occurs in nitrate reductase null mutants (Wang et al., 2004) which demonstrates that nitrate itself, and not the products of nitrate assimilation, causes the effect.

1.5.2 Nitrate regulates root development

Nitrate is also responsible for the regulation of lateral root development (Drew et al., 1973). Lateral root elongation enables the plant to forage through the soil for nitrate-rich patches. Since soil nitrate is present at micromolar to millimolar concentrations (Crawford and Glass, 1998), this is an essential mechanism in order to maximise nitrogen uptake. At zero, or low concentrations of nitrate, Arabidopsis lateral root growth is stimulated in order to forage for nitrate. Conversely, It has been demonstrated that the growth of lateral roots is inhibited when roots are grown in media containing a high nitrate concentration and this growth inhibition is maintained in nitrate reductase mutants, which again suggests that nitrate itself

is the signal for the inhibition (Zhang et al., 1999). However, in split root experiments in which *Arabidopsis* roots are grown half in high nitrate and half in nitrate-free media, lateral root growth is stimulated in the high nitrate compartment and inhibited in the compartment without nitrate (Ruffel et al., 2011). This is indicative that rather than responding exclusively to the local nitrate signal, the split-roots are also responding to a systemic signal of the overall nitrogen-status of the plant to maximise nitrogen uptake in an overall nitrogen-deficient plant. In other words, in a nitrogen-limited plant, the availability of external nitrate stimulates lateral root formation to maximise exploitation of the nutrient resource. Cytokinin synthesis mutants used in the split root experimental system were impaired for the systemic signalling of nitrogen demand and the addition of exogenous cytokinin recovered the wild type phenotype (Ruffel et al., 2011), which suggests that cytokinins may mediate this root-shoot-root systemic signalling of nitrogen-demand.

Auxin has been proposed to play a role in the long distance shoot to root systemic signalling of nitrogen. Ordinarily, *Arabidopsis* seedlings grown on high nitrate show inhibition of lateral root development, which results in short lateral roots in which growth is arrested just after emergence from the primary root. In NR-deficient mutants, this effect is enhanced, which indicates that nitrate itself elicits the response (Zhang et al., 1999). When *Arabidopsis* seedlings are transferred from a high to a low concentration of nitrate, lateral root growth is stimulated, which is accompanied by a 50% increase in root IAA (indole 3-acetic acid) levels (Walch-Liu et al., 2006a), which suggests a role for auxin in long distance systemic nitrogen signalling. The NRT1.1 transporter has been demonstrated to transport auxin, competitively with nitrate. When nitrate is present, auxin transport by NRT1.1 is inhibited and auxin builds up at the lateral root tips, thus promoting growth (Krouk et al., 2010).

1.5.3 Nitrate regulates shoot development

Plants use multiple ways to sense their continually changing nitrogen-status in order to adapt to changes by modulating enzyme activity and gene expression. Nitrate itself and the

phytohormone cytokinin appear to be important in mediating root to shoot signalling of nitrogen-status (Sakakibara, 2003). Nitrate-specific signalling refers to a series of specific changes caused by the nitrate ion and cytokinin-mediated nitrogen signalling encompasses nitrogen-dependent cytokinin accumulation (Sakakibara, 2003). Shoot development also appears to be regulated by nitrate induction of cytokinin-mediated growth, as evidenced by the fact that an increase in available external nitrate results in an increase in shoot size, which is accompanied by an increase in leaf cytokinins (Wagner and Beck, 1993; Walch-Liu et al., 2000; Yong et al., 2000; Rahayu et al., 2005). Further investigation revealed that *AtIPT3* (*A.thaliana* IsoPentenyl Transferase 3), a rate-limiting enzyme in cytokinin biosynthesis, is upregulated by nitrate, but not ammonium and that this increase in *AtIPT3* mRNA levels was maintained in detached roots and shoots (Miyawaki et al., 2004). Such a result suggests that cytokinins are responsive to external nitrate availability and may be involved in long-distance shoot to root nitrate signalling. Conversely, supply of ammonium or nitrogen deprivation rapidly inhibits shoot growth and is preceded by a decrease in leaf cytokinins in young leaves and xylem exudates (Sattelmacher and Marschner, 1978; Palmer et al., 1996; Rahayu et al., 2005).

1.6 Nitrate transport and its regulation

In order to exploit soil nitrate concentrations that can vary from micromolar to millimolar concentrations (Jackson and Caldwell, 1993), plants have both high affinity transporters (HATs) and low affinity transporters (LATs) for nitrate in their plasma membranes. The NPF (NRT1/PTR) family in Arabidopsis is a large family containing 53 nitrate and peptide transporters. To date, nitrate transport activity has been demonstrated for 16 out of 53 NPF proteins, however, in addition to nitrate, some NRT1 proteins, such as NRT1.1 have also been demonstrated to transport amino acids, peptides, glucosinolates, auxin and abscisic acid (ABA) (Zhou et al., 1998; Krouk et al., 2010; Kanno et al., 2012; Nour-Eldin et al., 2012). All NRT1 members characterised thus far have a low affinity for nitrate, except for

NRT1.1 which has a dual affinity for nitrate (Liu et al., 1999; Liu and Tsay, 2003). The NRT2 family contains seven members and all those characterised to date are high affinity transporters. Unlike the NRT1 family, only nitrate was identified as a substrate for NRT2 proteins.

High affinity nitrate transport activity is rapidly induced by the presence of exogenous nitrate (Crawford and Glass, 1998; Daniel-Vedele et al., 1998). High affinity nitrate uptake usually peaks within several hours post nitrate induction, then declines significantly potentially due to feedback inhibitory effects arising from the accumulation of nitrogen metabolites (Muller, 1992; Atilio J. and Causin, 1996). The induction of nitrate transport activity by exogenous nitrate is accompanied by an induction in nitrate transporter transcripts. For example, upon exposure of soy bean, tobacco and Arabidopsis roots to nitrate, NRT2.1 mRNA increases rapidly, reaching a peak after one hour (Amarasinghe et al., 1998; Krapp et al., 1998; Filleur and Daniel-Vedele, 1999). In addition to regulation by nitrate availability, nitrate transport is also regulated by amino acid and sugar levels. Increased amino acid availability has been linked to a decrease in nitrate transport, as well as a decrease in nitrate transporter transcripts (Muller et al., 1995; Nazoa et al., 2003). Whereas increases in sugar availability have been linked to increases in nitrate uptake transcripts and nitrate uptake, as demonstrated (de Jong et al., 2014).

The assimilation of nitrate requires carbon skeletons and thus nitrate uptake must be coordinated with the carbon status of the plant. Sugars produced via photosynthesis induce nitrate uptake as well as nitrate assimilatory enzymes such as nitrate reductase and nitrite reductase (Wirth et al., 2007; Lillo, 2008). Regulation by carbon availability adds another layer of complexity and control to the regulation of nitrate uptake and will be discussed later in this introduction.

The dual affinity transporter, NRT.1, has a role in regulating the abundance of *NRT2.1* transcripts in response to the nitrogen-demand status of the plant (Munos et al., 2004).

Ordinarily, supplying ammonium or glutamate to plant roots reduces the abundance of the *NRT2.1* transcript. However, this suppression is absent in *chl1-5* mutants which are deficient in the *NRT1.1* transcript. This suggests that NRT1.1 has an important role in sensing the plant nitrogen-demand and relaying this signal to suppress nitrate uptake when nitrogen demand is low.

1.7 NRT1.1 and its function as a nitrate transporter

NRT1.1 is the only nitrate transporter which has been demonstrated to operate in both high- and low-affinity transport modes. Nitrate uptake in roots of *Arabidopsis nrt1.1* deletion mutants (*chl1-5*), which have no detectable *NRT1.1* transcript (Tsay et al., 1993), were shown to be deficient in both high and low affinity nitrate uptake. Further transport studies in *Xenopus oocytes* expressing *NRT1.1* confirmed the dual affinity function of this transporter with a low affinity K_m for nitrate of around 5 mM, and a high affinity K_m of around 50 μ M. NRT1.1 switches between low and high affinity modes by Calcineurin B-like protein interacting kinase (CIPK23) phosphorylating the threonine residue 101 (T101) (Liu and Tsay, 2003; Ho Cheng et al., 2009; Ho et al., 2009). In 2014, two research groups published two linked mechanisms that could explain how phosphorylation switches the NRT1.1 transporters affinity for nitrate. Sun *et al.* suggested that T101 phosphorylation affects the dimerization of NRT1.1 and that this dimerization itself alters affinity (Sun et al., 2014). Parker and Newstead engineered a mutant form of NRT1.1 in which T101 was replaced by aspartate (T101A), thus mimicking phosphorylation. Comparisons made between this mutant and wild-type revealed that T101A had an increased transport rate as a result of increased structural flexibility and that the increase in K_m is the result of an increase in the rate of nitrate transport (Parker and Newstead, 2014). It is possible that both hypotheses are true and that increased nitrate uptake is the result of dimerization and increased structural flexibility.

1.8 Phosphorylated NRT1.1 regulates the high affinity induction of the primary nitrate response

In addition to functioning as a nitrate transporter, NRT1.1 has also been shown to be involved in regulating the PNR in response to nitrate availability (Ho et al., 2009). Wild-type plants exposed to increasing concentrations of nitrate show a biphasic induction of the primary nitrate response gene *NRT2.1*, with both a high and low affinity phase, reminiscent of the uptake biphasic response (Liu et al., 1999; Ho et al., 2009). In the *chl1-5* knockout mutant, this biphasic induction of *NRT2.1* is absent. However, these mutants are also deficient in nitrate uptake which raises the question of whether the loss of the PNR is due to a signalling defect or as a result of the reduced nitrate uptake. To answer this question, *chl1-5* EMS mutants were screened for the uncoupling of the sensory function from the uptake function (Ho et al., 2009). This screen returned the point mutation mutant *chl1-9* which was deficient in nitrate uptake, similar to the *chl1-5* mutant, but still showed the biphasic induction of *NRT2.1* consistent with NRT1.1 having a sensory function which is able to induce the PNR independent of its uptake function.

Phosphorylation of T101 controls the switch from low to high affinity uptake (Liu and Tsay, 2003). Transgenic plants expressing the T101D mutation, which mimics phosphorylated NRT1.1, induced monophasic *NRT2.1* expression in the high affinity range. Plants expressing the T101A mutation, which mimics dephosphorylated NRT1.1, induced biphasic *NRT2.1* expression in both the high and low affinity ranges, and to a greater extent than in wild-type plants. This confirms that phosphorylated NRT1.1, which is active for high affinity nitrate uptake, is responsible for signalling the availability of external nitrate in order to regulate the expression of *NRT2.1* through an as yet unknown mechanism. Experimental evidence, however, indicates that dephosphorylated NRT1.1, whilst able to function as a low affinity transporter, does not regulate low affinity induction of *NRT2.1*, suggesting that in this form, the protein is either unable to function as a sensor for nitrate, or that low affinity nitrate

sensing is controlled by a mechanism other than phosphorylation. This is contrary to the interpretation of Ho et al. and the majority of the literature which states that dephosphorylated NRT1.1 protein regulates low affinity nitrate sensing.

Support for the lack of a nitrate sensory role for dephosphorylated NRT1.1 is provided by the fact that a dephosphorylated form of NRT1.1 (T101A) was unable to attenuate the growth inhibitory effects of glutamate on roots upon supply of nitrate (Walch-Liu and Forde, 2008). Wild-type and transgenic plants constitutively overexpressing full-length NRT1.1 were able to relieve the growth inhibition caused by glutamate upon supply of nitrate. Nitrate uptake was not affected in the wild-type, full-length NRT1.1 line or T101A mutant, yet only the T101A mutant resulted in the inability to signal the availability of nitrate to antagonise glutamate inhibition of root growth. It is therefore likely that dephosphorylated NRT1.1 is defective for nitrate signalling or sensing.

1.9 Nitrate-regulated auxin transport by NRT1.1

NRT1.1-deficient mutants show decreased lateral root growth in nitrate-rich patches compared to wild-type plants, which suggests that the NRT1.1 transporter has a major role in the control of lateral root growth (Remans et al., 2006). Further work using *Xenopus oocytes* microinjected with NRT1.1 cRNA and supplied with exogenous labelled indole-3-acetic acid (IAA) showed that NRT1.1, as well as being a nitrate transporter, is also able to transport IAA, and that this uptake was inhibited by increasing nitrate concentrations (Krouk et al., 2010). Auxin accumulation in root tips stimulates lateral root growth (Benkova et al., 2003). This led to the proposal of a model for the control of lateral root growth by NRT1.1 (Figure 1.2).

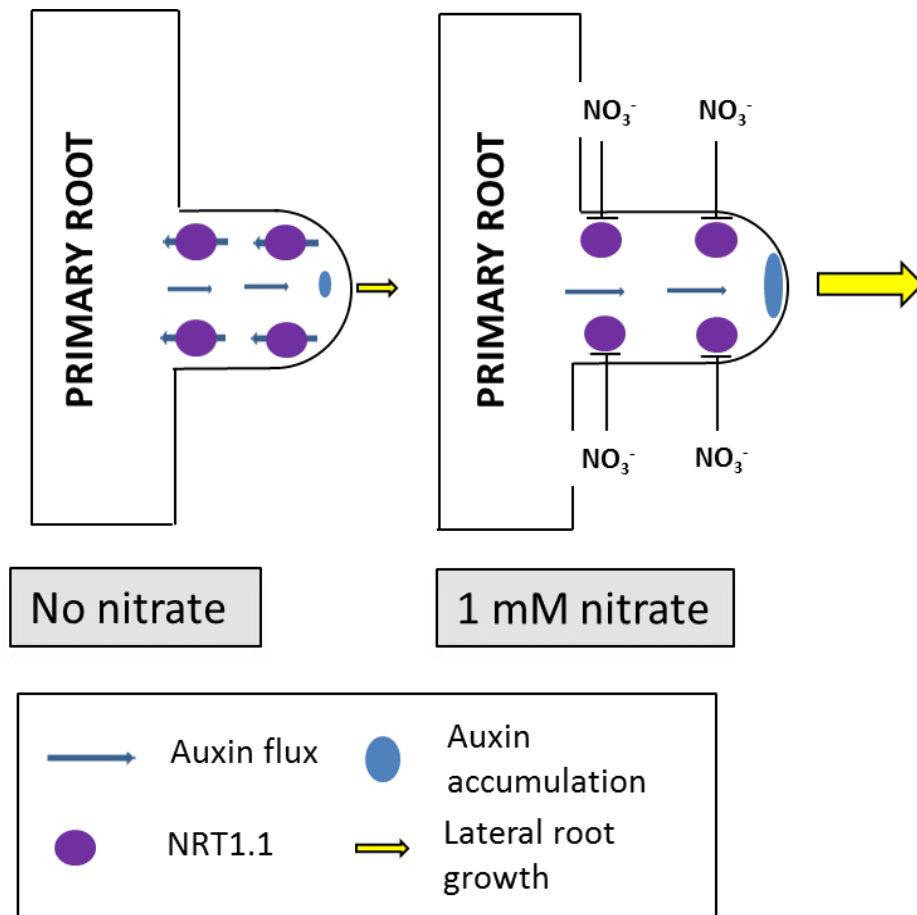


Figure 1.2: Regulation of lateral root growth by NRT1.1 in response to nitrate.

Auxin is transported to the lateral root tip, which in the absence of nitrate, is then transported away from the root tip by NRT1.1, thus preventing auxin accumulation and inhibiting lateral root growth. When nitrate is externally present, this inhibits the NRT1.1 mediated transport of auxin, resulting in accumulation at the root tip and subsequently influencing lateral root elongation.

1.10 miRNAs and the regulation of nitrogen metabolism

Micro RNAs (miRNAs) are endogenously transcribed, 21 – 24 nucleotide long molecules that are regulators of transcriptional, post-transcriptional and translational gene expression. Expression studies of plant miRNAs have recently revealed a number of differentially regulated miRNAs in response to nitrogen starvation. For example, the now well characterised miR169 and related family members in *Arabidopsis* have been shown to be

strongly down-regulated under nitrogen starvation conditions, and as a result, the expression of its targets, the *NFYA* transcription factor family, are strongly induced (Zhao et al., 2011). Additionally, constitutive expression of *miR169a*, which is one of the members of the miR169 family, resulted in hypersensitivity to nitrogen deprivation as determined by an observed increase in phenotypic traits associated with nitrogen starvation, such as yellowing of the leaves and a decrease in total nitrogen concentration. The decrease in total nitrogen was accompanied by a decrease in *NRT1.1* and *NRT2.1* transcripts, which suggests that miR169a has a role in regulating nitrate uptake, by regulating the transcript levels of *NRT1.1* and *NRT1.2* in response to nitrogen deprivation. Members of the miR169 family are also downregulated in maize (Xu et al., 2011; Zhao et al., 2012) and soybean (Wang et al., 2013) under nitrogen deprivation, suggesting that this may be a common feature of members of this family.

miRNAs have been associated with the regulation of root morphology in response to nitrogen starvation. In *Arabidopsis*, nitrogen limitation increases the expression of miR160 and miR171, and decreases the abundance of miR167 (Liang et al., 2012). It appears however, that regulation of root morphology by miRNAs may differ between species because, in maize roots expression of miR167a-d, g-l and miR164e-f are upregulated (Zhao et al., 2012) and miR167 is downregulated under nitrogen stress (Zhao et al., 2011). Common to both *Arabidopsis* and maize was the decreased abundance of miR167 transcript. The target for this family of miRNAs is the transcription factor, auxin-responsive factor 8 (ARF8), which inhibits lateral root formation. Further research demonstrated that miR167 was responsive to auxin in rice cell cultures. The presence of exogenous auxin caused increased levels of miR167 transcripts, which was accompanied by decreased levels of ARF8 (Yang et al., 2006). The reverse trend was true in the absence of auxin. This reveals a clear signal transduction pathway; auxin increased the production of miR167 transcripts, which in turn decreased the abundance of their target ARF8, thus alleviating inhibition of lateral root formation. Under nitrogen deprivation, root morphology dramatically

changes as lateral roots go forth to forage for nitrogen sources. This data suggests that the changes in root morphology in response to nitrogen limitation may be regulated by specific miRNAs, and that the miRNAs involved in this process may be species specific.

miRNAs add a further layer of complexity in the regulation of nitrogen metabolism. It is becoming increasingly more apparent that miRNAs have an important role in the regulation of transcripts involved in nitrogen metabolism, and as more research emerges, we will be able to piece together this important regulatory network.

1.11 Transcription factors and nitrogen metabolism

1.11.1 ANR1 and the regulation of lateral root formation

The root-specific MADS-box transcription factor ANR1 is involved in plant developmental nitrogen responses (Zhang and Forde, 1998). Plants with reduced *ANR1* expression were impaired for lateral root formation in response to localised nitrate supply. Further to this, it was later revealed that *ANR1* was induced by nitrogen limitation and repressed by nitrate supply (Gan et al., 2005). This suggests that ANR1 has a role in the regulation of lateral root growth in response to the nitrogen-status of the plant. More specifically, ANR1 induces lateral root growth under nitrogen limitation in order to forage for nitrogen, depending on the overall nitrogen status of the plant.

1.11.2 The effects of Dof1 on nitrogen metabolism

To improve nitrate assimilation, the plant-specific Dof1 transcription factor was overexpressed in *Arabidopsis* (Yanagisawa et al., 2004) and rice (Kurai et al., 2011). The Dof1 protein is involved in activating the expression of genes involved in the production of keto acids, which in turn are required for the assimilation of nitrogen into amino acids. Overexpression of Dof1 in *Arabidopsis* resulted in an increase in total plant nitrogen content by 30 % (Yanagisawa et al., 2004). Similarly, rice overexpressing maize Dof1 (*ZmDof1*),

grown under nitrogen limitation had increased total carbon and nitrogen levels, accompanied by significant increases in biomass (Kurai et al., 2011). Presumably, increasing the flux of carbon metabolism and providing increased availability of carbon skeletons had the knock-on effect of increasing nitrate uptake and assimilation to match the carbon availability. Collectively, the studies reveal that Dof1 may play an important role in coordinating carbon and nitrogen metabolism.

1.11.3 GATA transcription factors

GATA transcription factors are key regulators of nitrogen metabolism in yeast (Liao et al., 2008). Arabidopsis has 30 GATA transcription factors, including the *GATA*, *Nitrate-inducible*, *Carbon metabolism-involved* (GNC) transcription factor, which, as the name suggests has been shown to be induced by nitrate. Loss-of-function *gnc* mutants had reduced chlorophyll levels and significant reductions in transcripts associated with carbon metabolism (Bi et al., 2005). These observations suggest a role for GNC in regulating carbon metabolism.

In-depth analysis revealed that a distinct set of nitrogen regulated genes, transcription factors and networks were conserved in rice and Arabidopsis exposed to the same nitrogen treatment conditions (Obertello et al., 2015). Since rice and Arabidopsis are highly divergent phylogenetically, any conserved networks are likely to be of importance. By integrating transcription factor knowledge obtained from Arabidopsis with experimental and bioinformatic data obtained from rice, the authors of the study were able to identify potential conserved master transcription factors which are involved in the nitrogen regulation. Included in the master transcription factor findings were members of the TGA and HHO transcription factor families. These have been shown to be involved in the nitrogen response in independent experiments, which serves to validate the network model. Members of the HHO family have been demonstrated to be important in the integration of phosphate and nitrogen signals by repressing primary root growth in response to phosphate deficiencies, but only when nitrate is present (Medici et al., 2015). Whereas, members of the TGA family

have been shown to be responsive to nitrate and have been shown to directly regulate transcript levels of *NRT2.1* and *NRT2.2* (Alvarez et al., 2014). Clearly, the Obertello, *et al.* study indicates that there may be conserved master transcriptional regulators which serve to integrate and respond to a variety of nutritional signals. Since plants are sessile organisms, the ability to rapidly and efficiently respond to changes in nutrient availability is vital for survival. Master transcriptional regulators would allow the plant to process a multitude of nutritional information and act on this information to expend energy on exploiting the most necessary nutrients.

1.12 Trehalose-6-phosphate responsive SnRK1 also affects nitrogen assimilation

SnRK1 is a serine-threonine protein kinase homolog of yeast Snf1 and animal AMPK. SnRK1 activates genes involved in degradation processes as well as photosynthesis, and inhibits those involved in biosynthetic processes, thus, in doing so, regulates metabolism and growth in response to carbon availability (Baena-González et al., 2007). Increased levels of trehalose-6-phosphate inhibit SnRK1, which serves to promote biosynthetic reactions in growing tissues (Zhang et al., 2009). Interestingly, Zhang *et al.* discovered that no other sugars or sugar phosphates had the same inhibitory effect on SnRK1 indicating that trehalose-6-phosphate has a unique regulatory role on SnRK1.

Overexpression of *MhSnRK1* from pingyitiancha (*Malus hupehensis*) in tomato enhanced nitrate-uptake efficiency by 15-30% and reduced fruit ripening time by 10 days (Wang et al., 2012). This indicates that additional to its role in regulating carbon metabolism, SnRK1 also affects nitrogen metabolism. Indeed it has been demonstrated that, similarly to yeast, in plants, SnRK1 controls the post-translational modification of nitrate reductase, as evidenced in transgenic potato overexpressing *SnRK1* (Purcell et al., 1998; Sugden, 1999). This is yet more evidence associating SnRK1 with a role in altering nitrogen metabolism. Since carbon and nitrogen metabolism are intimately linked through the assimilation of amino acids, it could be that affecting one pathway will ultimately affect the flux through the other pathway.

Alternatively, It is possible that SnRK1 has functional roles in both carbon and nitrogen metabolism and may function to regulate both in response to carbon availability.

1.13 Amino acid sensing

1.13.1 The effect of exogenous amino acids on plant growth

The effect of exogenous amino acids on plant growth has been studied a number of times within the last century. In various experiments, amino acids, alone or in combination, have been shown to inhibit, stimulate, or have no effect on plant growth or plant cell growth when added to the growth medium (Rognes et al., 1986; Forlani et al., 1994; Bonner et al., 1996; Bonner and Jensen, 1997). There is an obvious lack of consistency between experiments, which may indicate that plant nitrogen-status contributes to the regulation of the response to exogenous amino acids. The response variation may also be due to the use of cell suspension cultures, different plant species, as well as varying concentrations of exogenous amino acids, often well above those that occur naturally in the environment. However, despite this variation, the most frequently observed phenotype was inhibition of plant cell growth in response to all exogenous proteinogenic amino acids, except glutamine (Bonner et al., 1996; Bonner and Jensen, 1997). Bonner et al used exponentially dividing cells of *Nicotiana glauca* in cell suspension cultures to observe their response to exogenously provided amino acids. The authors concluded that 19 amino acids inhibited cell growth and that this inhibition could be overcome by the addition of glutamine to the media. (Bonner and Jensen, 1997). This led to the proposal that there are potentially two different mechanisms that can explain the observed responses to exogenous amino acids. The first is the 'specific amino acid inhibition' response. In this response, inhibition is usually caused by the exogenously provided amino acid and is often relieved by another amino acid, thus indicating that the inhibition is caused by interfering with the endogenous biosynthesis of the latter amino acid (Bonner and Jensen, 1997). The second mechanism is the 'general amino acid inhibition' response and relates to growth inhibitory effects that can be overcome by

exogenous glutamine. It has been hypothesised that the glutamine to amino acid ratio is important in this response, with programmed cell death rates increasing when the concentration of a single amino acid is increased in relation to glutamine. It is important to note, however, that Bonner and colleagues used cell suspension cultures in all their experiments, which may have altered the behaviour of the cells in response to amino acids. In a similar experiment, 18 amino acids were added in turn to *Arabidopsis* seedling roots, with and without additional glutamine (Forde, 2014). The authors reported that all amino acids except alanine, aspartate and glutamine inhibited primary root growth, however in the presence of glutamine, the inhibitory effects of asparagine, glycine, isoleucine, proline, serine, threonine, tryptophan and valine on root growth were suppressed (Forde, 2014). Conversely, seven amino acids (arginine, cysteine, glutamate, leucine, lysine, methionine, phenylalanine), when supplied with additional glutamine, caused inhibition of primary root growth. This response completely differs from that observed by Bonner *et al.* in *N. silvestris* cell cultures and likely indicates the importance of studying the effects of amino acids on the whole system rather than an isolated cell culture.

1.13.2 Nitrogen-status sensing

The influx and assimilation of nitrate and ammonium is regulated according to the availability of- and demand for- internal nitrogen. In other words, the nitrogen-status of the plant regulates nitrogen uptake, in combination with information on the availability of external nitrogen. This has been demonstrated in *Glycine max* seedlings pre-treated with exogenous amino acids (Ala or β Ala or Arg) which show a significant reduction in the uptake of labelled nitrate compared to plants which were not pre-treated (Muller *et al.*, 1995). This has led to a general consensus that the overall nitrogen-status of the plant is sensed and subsequently uptake and assimilation is regulated by this balance between external nitrogen availability (nitrogen supply) and internal nitrogen availability (nitrogen demand). Since the end products

of nitrogen assimilation are amino acids, it is logical that these downstream nitrogen metabolites may be key signals of internal nitrogen-status.

1.13.3 Glutamate and glutamine sensing

All forms of nitrogen taken up by the plant are first assimilated into glutamine and then glutamate; consequently these can be considered the pivotal amino acids in nitrogen metabolism. It is therefore no surprise that both these amino acids have been implicated in nitrogen-status sensing. It was established that supplying exogenous glutamine to barley roots led to an increase in root tissue glutamine concentrations and a reduction in NR activity (Fan et al., 2006), which suggests that excess levels of this amino acid negatively feedback to inhibit further nitrogen assimilation.

Glutamine concentrations in bacterial cells signal the internal nitrogen-status of the cell through interaction with the PII protein. PII is modified by uridylylation in response to levels of glutamine and 2-oxoglutarate. The uridylylation state of PII protein dictates how it can interact with other regulatory proteins to control the transcriptional and post-transcriptional regulation of glutamine synthetase (GS) (Ninfa and Jiang, 2005). For example, when cellular nitrogen is in excess, and therefore glutamine levels are high and 2-oxoglutarate levels are low, the PII protein is found in a non-uridylylated and active state, which can then interact with GS adenylyltransferase (GlnE) to adenylylate GS which inactivates the enzyme and reduces nitrogen assimilation (Ninfa and Jiang, 2005). The reverse is also true. PII can also alter transcription via interaction with the histidine kinase nitrogen regulation protein (NtrB) (Ninfa and Jiang, 2005).

A PII homologue, GLB1, was identified in Arabidopsis as a nuclear encoded, chloroplast targeted protein (Hsieh et al., 1998; Moorhead and Smith, 2003). Orthologues of the bacterial PII protein targets NtrB and GlnE do not exist in plants, therefore GLB1 is unable to transcriptionally or post-transcriptionally regulate GS in response to glutamine concentrations using the same mechanisms as bacteria. However, GLB1 is known to

interact with *N*-acetyl glutamate kinase (NAGK) (Sugiyama et al., 2004; Chen et al., 2006), which is a key enzyme in arginine biosynthesis. Subsequently, Arabidopsis GLB1 knock out mutants display reduced NAGK activity and reduced arginine synthesis (Ferrario-Mery et al., 2006). Consequently indicating that PII is involved in the relief of arginine feedback inhibition by NAGK in Arabidopsis. In contrast to Arabidopsis and bacterial PII, the model green algae *Chlamydomonas reinhardtii* was shown not to relieve arginine feedback inhibition of NAGK (Chellamuthu et al., 2014). Eventually, the authors identified that the addition of glutamine to the medium rescues the PII-NAGK interaction and therefore the arginine feedback inhibition of NAGK. This is surprising as it is the first known example of the PII protein directly sensing glutamine. It is now understood that this unusual ability to directly bind and sense glutamine is due to a region in the C-terminus of the protein known as the Q loop (Fallon and Weng, 2014). Interestingly, the Q loop of Arabidopsis and other *Brassicaceae* contain a three amino acid deletion, which offers an explanation as to why these plants are unable to directly sense glutamine (Fallon and Weng, 2014).

Much like *C. reinhardtii*, the PII in the green algae *Chlorella variabilis*, was shown to activate NAGK in a glutamine-dependent manner (Minaeva et al., 2015). Providing exogenous glutamine prevented feedback inhibition of NAGK on arginine synthesis, and was thus accompanied by an increase in internal arginine levels (Minaeva et al., 2015). Exogenous glutamine, but not methionine-DL-sulfoximine (MSX), was observed to have an inhibitory effect on glutamine synthetase (GS) activity, suggesting that GS is responsive to internal glutamine concentrations and that glutamine is a negative effector of GS activity (Minaeva et al., 2015).

In the legume *Lotus japonicus*, the *LjGLB1* gene encoding the PII protein was characterised and investigated for any involvement in nodulation pathways (D'Apuzzo et al., 2015). It was discovered that the *LjGLB1* transcript was induced in root and leaf tissues during the light period and that this effect could be mimicked in the dark by the addition of 3% sucrose. Thus indicating that induction of the transcript was mediated by sensing of photosynthetic

products (D'Apuzzo et al., 2015). Further, the authors revealed that at least part of the transcriptional regulation was nitrogen-dependent. Nitrogen starvation conditions caused a rapid, but brief decrease in *LjGLB1* transcript levels followed by a gradual increase in root tissues. Such results indicate that *LjGLB1* is strongly regulated at the transcriptional level in response to both carbon and nitrogen availability. Lines expressing *35S:LjGLB1* were used to test the relationship between PII and nodule formation on high nitrogen, which is known to inhibit nodule development. Nodulation was significantly reduced in wild-type plants grown on high nitrogen when compared with wild-type plants grown on low nitrogen (D'Apuzzo et al., 2015). However, in *35S:LjGLB1* lines, this inhibitory effect on nodule formation under high nitrogen was almost abolished. Such a result likely implicates a role for the PII protein in legumes in regulating nodule formation in response to nitrogen-status.

Arabidopsis PII (GLB1), although unable to bind glutamine, can directly bind 2-oxoglutarate and ATP or ADP (Smith et al., 2003), which is similar to bacterial PII. The binding of 2-oxoglutarate is dependent on the earlier binding of ATP. It is likely that GLB1 is saturated with ATP *in vivo* as the chloroplast concentration of ATP is predicted to be well above the binding constant of GLB1 which will enable the uninterrupted binding of 2-oxoglutarate (Smith et al., 2003). Such binding studies may indicate the function of ATP/ADP as a PII effector molecule, offering an additional level of regulation that adjusts PII activity in accordance with energy status as well as a role for PII as a 2-oxoglutarate sensor. Although Arabidopsis PII cannot directly bind glutamine, it is likely that an alternative glutamine sensing mechanism exists within *Brassicaceae*. This is supported by evidence that Arabidopsis *GLB1* transcript is repressed by glutamine (Hsieh et al., 1998).

Although glutamate is well placed as a potential signal linking internal nitrogen status with nitrogen assimilation, numerous studies have highlighted the fact that in comparison to all other amino acids, levels of glutamate remain surprisingly constant throughout diurnal light/dark cycles. Because nitrogen assimilation and amino acid biosynthesis are highest during the first 6 hours of daylight and decline throughout the day to low levels at night, the

levels of most amino acids fluctuate diurnally. In contrast, glutamate concentration is relatively invariant during the diurnal cycle in the leaves of tobacco, potato and *Arabidopsis* (Geiger et al., 1998; Matt et al., 2001; Urbanczyk-Wochniak et al., 2005; Gibon et al., 2006). Glutamate is positioned centrally in amino acid metabolism, and indeed nitrogen metabolism. It is formed by the action of GOGAT, it is a substrate for GS and various aminotransferases and GDH catalyses its deamination. This central positioning of glutamate in the metabolic network may contribute to buffering of its concentration. Nevertheless, when internal glutamate concentrations are forced to increase by exogenous feeding of glutamate or 2-oxoglutarate, (Schneidereit et al., 2006) a decrease in NR activity is observed (Fritz et al., 2006), as well as an increase in transcripts for GS and glutamate dehydrogenase (GDH) (Masclaux-Daubresse et al., 2005). These changes in enzyme activity and transcript abundance indicate that glutamate concentrations can be sensed. The changes in NR enzyme activity, GS and GDH transcript abundance will decrease nitrogen assimilation, increase the flow of glutamate to glutamine production and increase the catabolism of glutamate respectively, all of which will serve to lower glutamate levels back to within the normal range.

Glutamate sensing and its subsequent signal transduction remains poorly understood. *Arabidopsis* has a family of 20 glutamate receptor (GLR) genes which show sequence similarity to mammalian ionotropic glutamate receptors (iGluRs) (Chiu et al., 2002). iGluRs in mammals are glutamate- and glycine-gated cation channels that mediate the uptake of K^+ , Na^+ , and Ca^{2+} into neurons (Dingledine et al., 1999). In plants, there is some evidence for the existence of glutamate-activated ion channels which are similar to mammalian iGluRs. In *Arabidopsis*, exogenously supplied glutamate inhibits primary root growth and stimulates lateral root growth at the primary root tip (Filleur et al., 2005; Walch-Liu et al., 2006b). Although *Arabidopsis* primary roots are inhibited by a number of other amino acids, only glutamate elicits a precise sequence of changes in growth, root tip morphology and root branching, indicating that the glutamate response is unique and specific (Filleur et al., 2005;

Walch-Liu et al., 2006b). Additionally, glutamate causes rapid membrane depolarisation in root cell plasma membranes, which is accompanied by a rise in intracellular Ca^{2+} (Demidchik et al., 2004). This glutamate-activated membrane depolarisation is sensitive to La^{3+} , a cation channel blocker, which supports the premise that glutamate is activating a cation transporter to allow the flux of Ca^{2+} across the membrane and therefore that plant GLR may be mediating glutamate signalling. There have been a number of studies on specific Arabidopsis GLR receptors that have brought about mixed results. AtGLR2.1 and AtGLR2.8 appeared to be non-functional when expressed in oocytes (Lacombe et al., 2001; Roy et al., 2008), whereas AtGLR3.7 was permeable to Na^+ , Ca^{2+} , and Ba^{2+} , but the transporters activity was insensitive to glutamate and glycine (Roy et al., 2008). Recently, two Arabidopsis glutamate receptors, AtGLR1.1 and AtGLR1.4 were confirmed to have functional ion pore domains which are permeable to Na^+ , K^+ and Ca^{2+} ions (Tapken and Hollmann, 2008). The glutamate-activated and AtGLR1.1 membrane depolarisations share similar properties; both are sensitive to La^{3+} , both are activated by glutamate and both induce the transport of the same ions. Unexpectedly, when *AtGLR1.4* was expressed in oocytes it was gated by 7 amino acids, yet insensitive to glutamate and glycine (Tapken et al., 2013). Similarly, when *AtGLR3.4* was expressed in human embryonic kidney cells, it was found to be gated by Asn, Ser and Gly, but only weakly by Glu (Vincill et al., 2012). These studies demonstrate that unlike mammalian iGluRs, plant GLR receptors have broad ligand specificity and may function similarly to iGluRs in mammals as a gated ion channel, the activity of which is mediated by ligand-binding activation. The mechanism for signal transduction following opening of the ion channel is unknown but it is possible that the ions transported, for example Ca^{2+} , could be the secondary messenger molecules responsible for transducing the glutamate signal.

A screen of >1500 molecules bioactive in *Saccharomyces cerevisiae* identified two groups of glutamate antagonists that interfered with the glutamate response (Forde et al., 2013). One of the small bioactive molecules, 2-(4-chloro-3-methylphenyl)-2-oxoethyl thiocyanate

(CMOT), is known to target Ste11, a conserved MAP kinase kinase. This led to the authors producing a triple knockout mutant, *mekk1 mekk2 mekk3* (*mekk1/2/3*) and discovering that root growth in this mutant was generally insensitive to glutamate. However, the roots of the *mekk1/2/3* mutant remained sensitive to inhibition by other amino acids (asparagine, cysteine, glycine and serine) (Forde et al., 2013). The sensitivity of the *mekk1/2/3* mutant to glutamate was restored by expressing the *MEKK1* gene in the triple mutant background. Thus demonstrating that *MEKK1* plays an important role in glutamate signalling which is a significant finding as it, for the first time in plants, provides evidence of the existence of a glutamate signalling pathway in plants.

Recently, serine was suggested to be a signal involved in regulating the transcriptional control of photorespiration-related genes in Arabidopsis (Timm et al., 2013). The mechanism behind this is yet to be elucidated, but it is interesting that in mammals, serine is a ligand and a potent agonist for the glutamate activated *N*-methyl-D-aspartate (NMDA) receptor, which is a type of glutamate receptor (Mothet et al., 2000).

1.14 Alanine aminotransferase

Alanine has not directly been implicated in nitrogen-status signalling but is rather linked to stresses such as hypoxia and drought (Good and Crosby, 1989; Ricoult et al., 2006; Miyashita et al., 2007). However, somewhat unexpectedly, manipulation of alanine metabolism through root-specific overexpression of *AlaAT* led to a high NUE phenotype in canola and rice (Good et al., 2007; Shrawat et al., 2008). These results are surprising because the *AlaAT* enzyme is not considered an integral part of nitrogen assimilation and to date it is unknown why overexpression of *AlaAT* results in this phenotype. Transgenic canola expressing barley *AlaAT1* (cytosolic isoform) under the control of a root-specific promoter, accumulate alanine in root tissue (Good et al., 2007). This suggests that the net flux of the *AlaAT* reaction is in the direction of alanine production. The tissue specificity of transgene expression appears to be important because the high NUE phenotype is not observed when

constitutive promoters such as CaMV35S are used to drive transgene expression (Good et al., 2007).

Although rice plants overexpressing barley *AlaAT1* under the control of a root-specific promoter also showed a high NUE phenotype, they exhibited no alanine accumulation, but did show significant increases in glutamine, glutamate and asparagine. This suggests that in rice the net flux of the AlaAT reaction is in the direction of glutamate production. However, it is worth noting that canola metabolites were measured on plants grown on low N (0.5 mM NO_3^-), whereas rice metabolites were measured on plants grown at high N (4 mM NH_4^+). These inconsistencies could alter metabolite content since it is well known that different N sources and concentrations induce different metabolic responses in plants. It would be interesting to see whether these metabolite measurements hold true if the transgenic rice was grown on the same low N source as the canola plants.

AlaAT catalyses the reversible reaction in which the amine group from glutamate is transferred to pyruvate to form alanine and 2-oxoglutarate (Figure 1.3). Like most aminotransferases, the AlaAT reaction consumes carbon skeletons in the form of pyruvate or 2-oxoglutarate and nitrogen in the form of the amino acids glutamate or alanine, and therefore is involved in both carbon and nitrogen metabolism. The high NUE phenotype produced by overexpressing this enzyme is not accompanied by an increase in transcripts relating to nitrogen uptake and assimilation in rice, which suggests that the changes occurring to produce the phenotype may be at the post transcriptional level rather than the transcriptional level (Beatty et al., 2009). Transgenic canola lines, however, show an increase in transcripts for NRT2.1 and NRT2.2 (high affinity nitrate uptake) so perhaps transcript abundance requires further investigation. Although nitrogen metabolism related transcripts are not differentially expressed, total nitrogen content is increased, suggesting that the high NUE phenotype results from interfering with negative feedback inhibition of nitrogen uptake or assimilation. It could be one of the direct products of the AlaAT reaction; alanine, glutamate, 2-oxoglutarate or pyruvate that provides a signal that promotes growth

by interfering with feedback mechanisms at low nitrogen. Alternatively, the signalling mechanism could arise from the wider metabolic network; from metabolites indirectly related to the AlaAT reaction such as glutamine. The decrease in shoot glutamine concentrations in the transgenic canola plants compared to wild-type certainly suggests this metabolite may be a signalling candidate. Added to this observation, the evidence that glutamine levels are potentially sensed in plants makes this metabolite an ideal signalling candidate to investigate further.

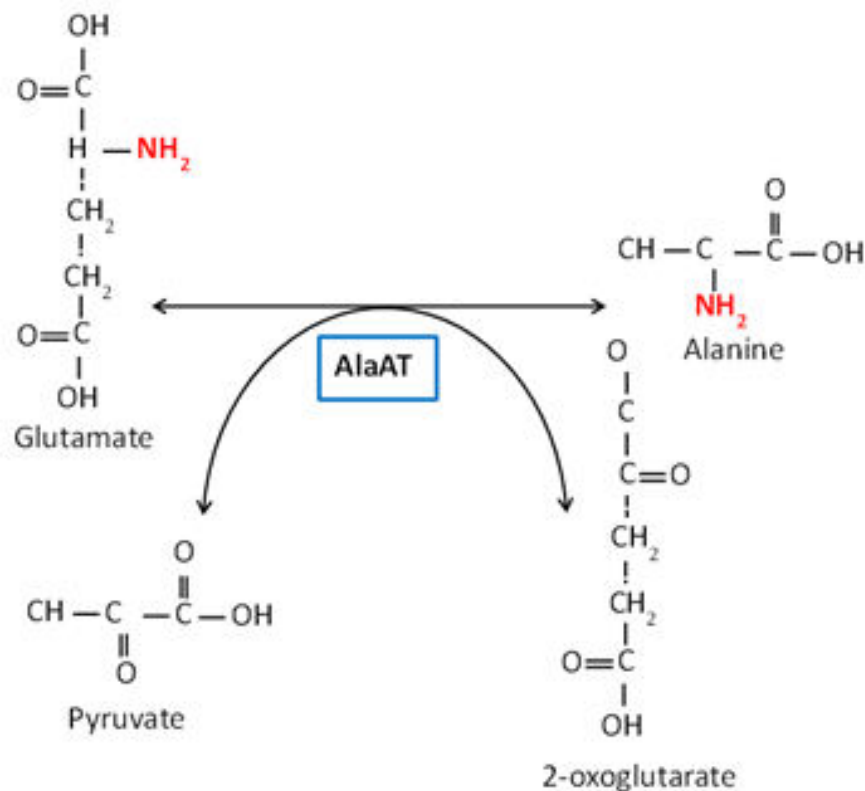


Figure 1.3. Alanine aminotransferase reaction.

This enzyme catalyses the reversible transfer of the amino group from alanine to 2-oxoglutarate to produce glutamate and pyruvate.

1.15 Amino acid transporters and nitrogen-status sensing

Currently, the mechanism for plant nitrogen-status sensing is poorly understood. However, there is evidence in animals that an amino acid transporter, PATH, is involved in the sensing of internal amino acids by acting as a transceptor (Goberdhan et al., 2005; Sancak et al., 2008). The PATH transceptor detects nutrient availability (in the form of amino acids) and interacts with the TOR signalling pathway to ultimately modulate growth. What is interesting about PATH is that it has a low transport capacity, yet it is still able to activate the downstream target of TOR, S6K, as demonstrated by *in vitro* oocyte assays when exogenous alanine is supplied, which further supports its role as a transceptor (Goberdhan et al., 2005). In mammals, TOR is activated when it is associated with the multi-protein complex TORC1 (which consists of TOR, KOG1/RAPTOR and LST8/GbetaL) or TORC2 (which consists of TOR, LST8/GbetaL and AVO3/RICTOR). More recent work has identified human member 9 of the solute carrier family 38 (SLC38A9) as a putative amino acid transceptor in humans (Rebsamen et al., 2015). This transporter localises to the lysosomal membrane, where it interacts with, and regulates, mTORC1 activity in response to amino acid availability. Since SLC38A9 has a low transport capacity, it is likely that it functions to sense amino acid availability by binding rather than transporting them. In Arabidopsis, all components of TORC1 are conserved (Mahfouz et al., 2006), making it plausible that the same TOR pathway could be responsive to amino acid availability and function to modulate growth in plants.

Further examples of amino acid sensors in yeast include the general amino acid permease (Gap1) and the SPS sensor. The extremely inefficient plasma membrane amino acid transporter Ssy1 operates in conjunction with two peripheral membrane proteins, Ptr3 and Ssy5, to form the SPS sensor (Forsberg et al., 2001; Forsberg and Ljungdahl, 2001; Hundal and Taylor, 2009). The binding of amino acids triggers a conformational change in Ssy1, which activates the SPS sensor. Ssy5, which is a putative chymotrypsin-like serine

protease, exhibits proteolytic activity upon the activation of the SPS sensor, which leads to the endoproteolytic processing of the N-terminals of the transcription factors Stp1 and Stp2 (Forsberg et al., 2001). The shorter forms of Stp1 and Stp2 are then able to efficiently target to the nucleus where they bind SPS sensor regulated promoters to induce transcription of transcripts including those associated with amino acid and peptide transport (Forsberg and Ljungdahl, 2001). Essentially, amino acid uptake in to the cell is regulated by the external availability of amino acids.

Gap1 is an amino acid transporter in yeast which transports all naturally occurring proteinogenic L-amino acids, D-amino acids and amino acid analogues (Grenson et al., 1970; Jauniaux and Grenson, 1990). There is convincing evidence that this transporter plays a role as an amino acid sensor, inducing the activation of Protein Kinase A (PKA) signalling upon the addition of amino acids to cells starved for nitrogen on glucose medium (Donaton et al., 2003; Holsbeeks et al., 2004). In order to enter the exponential growth phase, yeast requires a fermentable carbon source, as well as a complete nutrient medium, i.e. containing nitrogen, phosphate and sulphate. This so-called 'fermentable growth medium-induced' (FGM) pathway is active only when the growth medium contains all these essential nutrients. If just one of these essential nutrients, for example nitrogen, is absent, cell cycle arrest occurs and yeast enters the stationary growth phase and PKA targets, such as trehalase are repressed. Upon supplying amino acids to these nitrogen starved yeast cells, an upregulation of PKA signalling is observed and is accompanied by an increase in activity of PKA targets (Conrad et al., 2014). The isolation of two constitutively PKA activating alleles; one C-terminal truncation of 14 amino acids (Gap1 Δ C6) and one 26 amino acids (Gap1 Δ C9), provided strong evidence for the amino acid sensory function of Gap1 and its ability to trigger PKA signalling. In both of the shortened alleles, L-citrulline transport was at least equal to wild-type levels, yet the ability of this amino acid to induce trehalase activity was significantly reduced (Donaton et al., 2003). The uncoupling of the sensory role from the transport function of this protein demonstrates its ability as a transceptor.

1.16 Aims and Objectives of the work undertaken

The aim of the work described in this thesis was to elucidate the mechanisms by which *Arabidopsis* plants are able to sense their nitrogen-status. It is clear that plants can respond to altered concentrations of exogenous and intracellular amino acids, but the sensing and signalling mechanisms have not yet been elucidated. The objective of the research was to use a reverse genetics to screen to identify candidate genes involved in nitrogen-status signalling. Based on evidence for the existence of amino acid transceptors (i.e. transporters with a signalling function) in *Drosophila* and yeast, this project will focus on amino acid transporters as candidates for involvement in nitrogen-status sensing.

The secondary objective was to characterise the function of the identified candidate genes with respect to their potential involvement in nitrogen status signalling.

Chapter 2: Materials and methods

2.1 Reagents

All reagents were from Sigma-Aldrich, Dorset, UK, unless otherwise stated. All primers used were salt-free and ordered at 100 μ M in H₂O from Eurofins, London, UK.

2.2 Growth assay

2.2.1 Germination

Approximately 0.2 ml *Arabidopsis* (ecotype Col-0) seeds were surface sterilized by incubating in 10% (v/v) sodium hypochlorite, available chlorine 10-15%, for 10 minutes before being washed six times with sterile distilled water. Individual seeds were pipetted onto plates containing 0.44% (w/v) Murashige and Skoog medium (including vitamins) (Duchefa Biochimie, Haarlem, Netherlands) with 0.04% (w/v) MES, 1% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8). Sown seeds were kept in the dark at 4°C for 48 hours, and then transferred to the growth chamber, with a diurnal cycle of 16 h light at 20°C and 8 h dark at 18°C. After 11 days, seedlings were transplanted in to the soilless medium.

2.2.2 Soilless media

11 day old seedlings were transplanted in to either 1) 50% sand (v/v), 30% fine (v/v) and 20% coarse vermiculite, or 2) 50% (v/v) peat and 30% (v/v) fine and 20% (v/v) coarse grained vermiculite, or 3) rockwool plugs (Grodon, West Sussex, UK). Trays of 12 and 24 plants were placed in a growth chamber, with diurnal cycles of 16 h light at 20°C and 8 h dark at 18°C.

2.2.3 Nitrogen sources

100 ml peat was supplemented with 260 mg K₂HPO₄, 396 mg GRANUKAL 85 (80% (w/v) CaCO₃ & 5% (w/v) MgCO₃ – Kreidewerke Dammann KG, Soehle, Germany), 1.6 mg

Fetrilon-Combi micronutrient fertiliser (BASF AG, Ludwigshafen, Germany) and 30 ml tap water. Sand and Rockwool plugs were watered with a modified nitrogen-free Hoagland's solution (0.5 mM KH_2PO_4 , 2.5 mM KCl, 2.0 mM CaCl_2 , 1.0 mM MgSO_4 , 1.0 mM MES (pH 5.8), 50 μM FeEDDHA (pH 7.7), 50 μM H_3BO_3 , 10 μM MnSO_4 , 1.0 μM ZnSO_4 , 1.0 μM CuSO_4 and 0.5 μM Na_2MoO_4) plus the indicated additional concentrations of nitrogen.

2.3 SALK and SAIL genotyping

2.3.1 Seed bulking

SALK and SAIL T-DNA insertion mutant lines were obtained from the National Arabidopsis Stock Centre, Nottingham (<http://arabidopsis.info/>). Seeds were bulked up by sowing on standard greenhouse soil and collecting seed at maturity. For genotyping, lines were again sown on to standard greenhouse soil and leaf samples were taken from 18 – 30 day old plants and frozen in liquid N_2 .

2.3.2 Genomic DNA extraction

Genomic DNA was extracted from leaves of soil-grown SALK, SAIL and parental wild-type plants. Up to two small leaves were placed in a 2 ml Eppendorf tube together with a glass grinding ball and the tissue was ground to powder using the Qiagen TissueLyser. 400 μL of extraction buffer (200 mM Tris-Cl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) were added to each sample followed by thorough vortexing. The sample was allowed to equilibrate to room temperature and centrifuged at 16,000 $\times g$ in a microcentrifuge (Eppendorf) for one minute. 300 μL of the supernatant were transferred to a fresh tube and the same volume of isopropanol added. DNA was allowed to precipitate at room temperature for five minutes before centrifugation at maximum speed for five minutes. The pellet was dried in an Eppendorf vacufuge concentrator at 60 $^\circ\text{C}$ and taken up in 100 μL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) without additional mixing. The DNA was diluted 10 times in TE buffer to be used as a template in PCR reactions

2.3.3 Genotyping PCRs

For genotyping PCRs, 5 µl 10x diluted gDNA was used in an 18 µl reaction of MegaMix Blue, Mastermix (Microzone, UK); with 1 µl 10 µM forward primer and 1 µl 10 µM reverse primer. A list of the primers used to genotype each line can be found in the Appendix A Table 1. WT gDNA was included as a positive control and no-template controls were used as negative control. PCR was performed at an annealing temperature of ~60°C with 30 cycles.

2.4 Metabolite analysis

2.4.1 GC-MS sample preparation

Samples were prepared for GC-MS based on Lisec *et al.* (2006). Frozen samples were ground to a fine powder using the Qiagen TissueLyser then dissolved in 1400 µl 100% methanol (HPLC grade) and vortexed. 60 µl 0.2 mg/ml Norvaline in water was added as an internal standard. The samples were heated for 10 min at 70°C with shaking at 950 rpm then centrifuged for 10 min at 1100 xg. 750 µl chloroform (HPLC grade) was added and mixed with 1500 µl dH₂O before being centrifuged for 15 min at 2200 xg. 150 µl of the polar phase was dried in an Eppendorf Vacufuge Concentrator (Fischer Scientific, UK), overnight for derivitisation. Dried samples were suspended in 40 µl of 20 mg ml⁻¹ methoxyamine hydrochloride in pyridine (HPLC grade) and incubated for 2 hours at 37°C with shaking at 900 rpm. For samples to be analysed using Tagfinder, 10 µl of an alkane mix containing linear alkanes of 10, 12, 15, 18, 19, 22, 28, 32 and 25 C, each at 22 µg ml⁻¹, was added as retention time standards to allow the identification of analytes simultaneously using TagFinder (Luedemann *et al.*, 2008). 70 µl MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (HiChrom, Berkshire, UK)) was added to each sample and incubated for 30 minutes at 37°C at 900 rpm. Samples were transferred to glass vials (Chromacol, Welwyn garden city, UK) with a septum cap for injection.

2.4.2 GC-MS analysis

GC-MS analysis was carried out using an Agilent 79890 GC coupled to an Agilent 5975 quadrupole MS detector (70 eV). The Agilent autotune method was used to calibrate the mass spectrometer prior to each run. 1 μ l of sample was injected each time. During the run, the oven was heated to 70°C for 5 min, then increased at a rate of 5°C/min until a temperature of 280°C. The maximum temperature was held for 3 minutes before being decreased to 70°C at 120°C/min with a helium gas flow of 0.6 ml min⁻¹ and a solvent delay of 10 minutes. Mass spectra were acquired using a scan and/or single ion monitoring (SIM) method.

Baseline correction using MetAlign was performed on all GC-MS data before analysis was started. Elution times and mass fragments of amino acids were determined by comparison with amino acid standards. The GC peaks and ion abundances of each metabolite were obtained using Chemstation software (Agilent Technologies, Wokingham, UK) and expressed relative to the internal Norvaline standard. Concentrations of amino acids were calculated by reference to a standard curve.

To determine the metabolic profile of mutant lines and WT in Chapter 5, a broad-spectrum GC-MS metabolic profiling approach was employed using TagFinder software (Luedemann et al., 2008). TagFinder functions by aligning chromatograms from multiple samples based on the elution times of alkanes of varying masses which are included as retention time references during the derivatisation process. Once chromatograms are aligned, the software can then group analytes together based on retention time. The automated identification of analytes can then occur simultaneously in all samples based on the analytes retention time and mass spectrum. After atomisation, the mass spectrum for each identified analyte is then referenced, by eye, to the corresponding mass spectra for that analyte in the NIST library.

2.5 Transcript analysis

2.5.1 Plant growth conditions

Arabidopsis seeds were sterilised and germinated on plates, as described in 2.2.1. After 11 days, seedlings were removed from plates and placed in 25 ml conical flasks containing 5 ml 0.1 mM CaCl_2 for 24 hours in order to starve them of nitrogen. After 24 hours, the starvation solution was removed and 5 ml N-free Hoagland's solution plus, either 1 mM NH_4NO_3 (untreated control) or 1 mM NH_4NO_3 + 1 mM Ala was added to each flask. Root tissue was sampled after 24 hours and frozen in liquid N_2 for qRT-PCR analysis.

2.5.2 RNA extraction

Frozen samples were ground to a fine powder using the Qiagen TissueLyser. Total RNA was isolated by adding 1 ml Trizol reagent (Life Technologies, UK) to each frozen sample, then acclimating to room temperature whilst vortexing. Samples were then centrifuged at 12,000 xg for 10 minutes at 4°C. Approximately 800 μl supernatant was removed and 400 μl chloroform:isoamylalcohol (24:1) was added to the samples, which were then vortexed and allowed to stand at room temperature for 5 minutes. Samples were then centrifuged at 12,000 xg for 15 minutes at 4°C. The aqueous layer was transferred to a fresh Eppendorf tube and an equal volume of isopropanol was added. Tubes were inverted and left to precipitate for 10 minutes at room temperature. Samples were then centrifuged at 12,000 xg for 10 minutes at 4°C to pellet the RNA. RNA was washed with 1 ml 70% (v/v) EtOH (in DEPC-treated H_2O). Tubes were inverted 3 times then centrifuged at 12,000 xg for 5 minutes at 4°C. The supernatant was discarded and the pellet was dried in an Eppendorf vacufuge concentrator for approximately 2 hours. The pellet was resuspended in 50 μl DEPC-treated H_2O and incubated for 10 minutes at 50°C.

2.5.3 RNA DNase treatment

After extraction, RNA was treated with DNase to prevent DNA contamination. 1 µl DNaseI reaction buffer (Life Technologies, UK), 2 µl DNaseI (Life Technologies, UK), and 0.5 µl RNase Inhibitor (Life Technologies, UK) were added to 6.5 µl RNA and incubated for 30 minutes at 37°C. The DNaseI reaction was terminated by adding 2 µl 25 mM EDTA and incubating for 10 minutes at 75°C. RNA quantity and purity was determined using a NanoPhotometer P360 (Geneflow, Staffs, UK).

2.5.4 First strand cDNA synthesis

The first strand cDNA fragment was synthesized from DNaseI treated total RNA using RevertAid reverse transcriptase (Life Technologies, UK). 1 µl DNaseI treated RNA was added to a mastermix containing 1 µl Oligo (dT)₁₈ primer, 15 µl DEPC-H₂O, 4 µl 5x reaction buffer, 1 µl RNase inhibitor, 2 µl 10 mM dNTPs and 1 µl reverse transcriptase. Reactions were incubated for 60 minutes at 42°C, followed by 5 minutes at 70°C to allow first strand cDNA synthesis.

2.5.5 qRT-PCR analysis of transcripts

11 pairs of primers were designed to amplify 11 target transcripts (Table 2.1). Where possible, primers were designed to span intron/exon boundaries to avoid amplification of gDNA in qRT-PCR. qRT-PCR was performed with Applied Biosystems 7300 Real-Time PCR System (Life Technologies, UK) in a final volume of 25 µl containing 2 µl of a 1/10 dilution of cDNA in water, 12.5 µl SYBR Green Select Mastermix (Life Technologies, UK), 10 µM forward and reverse primers. The thermal cycling conditions were as follows: 40 cycles of 95°C for 15 seconds for denaturation and 60°C for 1 minute for annealing and extension. qRT-PCR was performed on 3 biological replicates, which were each run in duplicate on the plate. Negative no-template controls were included on each plate for each primer pair used.

The Arabidopsis TIP41-like transcript was used to normalise transcript abundance as this was shown to be a very stable reference transcript (Czechowski et al., 2005). Transcripts were expressed relative to either wild-type or untreated controls (minus alanine addition in the media). Primer efficiency was determined using a standard curve created by a series of 2-fold cDNA dilutions and using the equation:

$$\text{Efficiency} = 10^{(1/\text{slope})}$$

All primer efficiencies were calculated to be between 1.9 and 2.1. The relative transcript abundances were calculated using the following equation:

$$\text{NRQ} = E_1^{(C1-T1)} / E_2^{(C2-T2)}$$

Where NRQ is the normalised relative quantity, E_1 is primer efficiency 1, E_2 is primer efficiency 2, C1 is control treatment 1, C2 is control treatment 2, T1 is treatment 1 and T2 is treatment 2.

2.6 Qualitative RT-PCR of SALK lines

RT-PCR was used to check for transcripts in SALK T-DNA insertion mutants isolated from the reverse genetic growth-response screen in Chapter 4 in order to determine whether they were null mutants. Whole mutant seedlings were harvested from plates (see 2.2.1 for plate set up) at 11 days after sowing. RNA extraction, DNaseI treatment and cDNA synthesis were as described in 2.5.2, 2.5.3 and 2.5.4, respectively. Primers were designed to span the full length of the transcript (Table 2.2).

Table 2.1 List of primer pairs for qRT-PCR

Target name	Primer name	Sequence	Length	Tm	Start (bp)	Stop (bp)	Product length	GC %	Exon junction	Blast ?	Acession no
TIP41-like protein	TIP4_F	GCGATTTTGGCTGA GAGTTGA	21	59.1 9	711	731	175	47.62	exon-6/exon-7	OK	AT4G34270
	TIP4_R	GTCGCTCCAGACTG CTAAGT	20	59.4 7	885	866	175	55	exon-7/exon-8	OK	AT4G34270
Nitrate transporter 1.1	NRT1.1_F	TTGGGCAACATGCA TCCTCT	20	59.9 6	1035	1054	104	50	No	OK	AT1G12110.1
	NRT1.1_R	GATCTCGAAGCTCC CGATG	20	60.0 4	1138	1119	104	60	No	OK	AT1G12110.1
Nitrate transporter 1.2	NRT1.2_F	CGCCTCTTTCGTCT TAGTGGT	21	60.0 7	158	178	195	52.38	exon-2/exon-3	OK	AT1G69850.1
	NRT1.2_R	GTGGAGAAGAAAG CGTCGGA	20	60.0 4	352	333	195	55	exon-3/exon-4	OK	AT1G69850.1
Nitrate transporter 2.1	NRT2.1_F	CCGAGTACTTCTTT GACAGGTTTC	24	59.5 5	971	994	181	45.83	exon-2/exon-3	OK	AT1G08090.1
	NRT2.1_R	CCACCAGCCGTCTG TATGAT	20	59.5 3	1151	1132	181	55	No	OK	AT1G08090.1
Nitrate transporter 2.2	NRT2.2_F	GTAGTGAGCCGAC CAACACA	20	59.9 7	80	99	125	55	No	OK	AT1G08100.1
	NRT2.2_R	GATCCACGAGAGGT GGAACG	20	60.1 8	204	185	125	60	No	OK	AT1G08100.1

Table 2.1 cont. List of primer pairs for qRT-PCR

Nitrate transporter 2.4	NRT2.4_F	TGAGTACTTTTTCG ACAGGTTCCA	24	60.1 4	909	932	192	41. 67	exon- 3/exon-4	OK	AT5G60770.1
	NRT2.4_R	AGGCAGAAGAAACC GCCTAA	20	59.3 1	1100	1081	192	50	No	OK	AT5G60770.1
Nitrate reductase 2	NR2_F	CGACGAAGAAGGTT GGTGGT	20	60.2 5	1125	1144	199	55	exon- 1/exon-2	OK	AT1G37130.1
	NR2_R	TCCACCATCTACCG TGACCT	20	59.9 6	1323	1304	199	55	exon- 2/exon-3	OK	AT1G37130.1
Nitrate reductase 1	NR1_F	GGCTACGCTTATTC TGGAGGA	21	59.3 1	1258	1278	105	52. 38	exon- 2/exon-3	OK	AT1G77760.1
	NR1_R	CGGTTTCTCCTGGT GGTCAA	20	59.8 9	1362	1343	105	55	No	OK	AT1G77760.1
Nitrite reductase 1	NiR1_F	GAATCCACCGCCTC TGTTGA	20	60.0 4	131	150	134	55	No	OK	AT2G15620.1
	NiR1_R	ATGGGCTCTCCCTC GATCTT	20	60.1 1	264	245	134	55	No	OK	AT2G15620.1
Glutamine synthetase 1.2	GLN1.2_F	TGCGGCTGAGATCT TTGCTA	20	59.4 6	327	346	119	50	No	OK	AT1G66200
	GLN1.2_R	CACCAATGGGCCAT CCAAGA	20	60.3 2	445	426	119	55	No	OK	AT1G66200

Table 2.1 cont. List of primer pairs for qRT-PCR											
NADH-dependent glutamate synthase 1	GLT1_F	ACGAAGACGCCAA GCTACAA	20	59.9 7	973	992	152	50	No	OK	AT5G53460.
	GLT1_R	TCCTTTACGCCTGT GTGTCC	20	59.9 7	1124	1105	152	55	No	OK	AT5G53460.

Table 2.2 Primers for SALK line RT-PCR

Name	Sequence	Tm	Product size
CAT2-rtPCR_F	C A C C A T G G G G T T T T T G G T	59.03	1912
CAT2-rtPCR_R	C T A T G C C A A A G A A C C T T C A T G T T	59.55	
CAT8-rtPCR_F	C A C C A T G A T C C C T G C T T C A	60.64	1777
CAT8-rtPCR_R	T T A T C T C T C T C C C T C A A A C T T T G C	60.25	
CAT6-rtPCR_F	A T G G A G G T C C A A A G C A G C	55.56	1749
CAT6-rtPCR_R	C A C T T C A A T T A G C T C T T T C A T G A C T	58.25	
LHT9-rtPCR_F	A T G G T C T C T A G T T C T C C G G T T T C	60.02	1320
LHT9-rtPCR_R	A G C G T A G A A A C T A T A A G T A G A A G A A T C T G	57.85	

For RT-PCR, 2 µl cDNA was used in a 20 µl reaction of MegaMix Blue, Mastermix (Microzone, UK); with 10 µM target specific forward and reverse primers (Table 2.2). WT cDNA was included as a positive control and no-reverse transcriptase controls were used as a negative control. The Arabidopsis TIP41-like transcript was included as a loading control. PCR was performed at the annealing temperatures specified in Table 2.2 (~60°C) with 30 cycles.

2.7 Isotope feeding experiments

2.7.1 ³H-Alanine feeding experiments

Plants were germinated on ½ MS plates as described in 2.2.1. At day 11 after sowing, 10 seedlings were selected for each genotype and placed in a flask containing 5 ml of 0.1 mM CaCl₂ to starve them of all nutrients. After 24 hours, plants were removed, blotted dry and

resuspended in 5 ml of uptake medium (either 1 mM alanine or 1 mM NH_4NO_3 + 1 mM alanine). 0.038 MBq of ^3H -Alanine (Perkin Elmer, UK) was added and flasks were agitated at room temperature. Whole seedlings were sampled at 0 and 5 minute time points. Excess label was washed off by rinsing with 0.1 mM CaCl_2 , followed by blotting dry and recording weight. Plant tissue was ground to a fine powder in liquid N_2 and solubilised by adding 1 ml dichloromethane and incubating at 60°C overnight. 4 ml of Scintisafe 3 scintillation cocktail (Thermo Fisher Scientific, UK) was added and samples were incubated at room temperature for 1 hour before shaking vigorously and counting using a Tri-carb 2800TR scintillation counter (Perkin Elmer, UK).

After counting CPM was converted to DPM using the following equation:

$$\text{DPM} = \text{CPM}/\text{Efficiency}$$

Where detector efficiency for ^3H -Alanine was calculated by the manufacturer to be 33.9%. DPM was then converted to Curies (Ci) and Ci was converted to pmol using the isotope specific activity, which for ^3H -Alanine is 84.1 Ci/mM. Values were then divided by sample fresh weight to be expressed as $\text{pmol}^{-1}\text{mg FW}^{-1}$.

2.7.2 K^{15}NO_3 stable isotope feeding experiments

Plant growth and treatment was exactly as in 2.7.1. After a 24 hour starvation period, seedlings were removed from the flasks, blotted dry and resuspended in uptake medium (either 0.5 mM K^{15}NO_3 + 0.5 mM $(\text{NH}_4)_2\text{SO}_4$ or 0.5 mM K^{15}NO_3 + 0.5 mM $(\text{NH}_4)_2\text{SO}_4$ + 1 mM Ala). Whole seedlings were sampled at 0 and 5 minute time points. Excess label was washed off by rinsing with 0.1 mM CaCl_2 , followed by blotting dry and recording weight. Plant tissue was ground to a fine powder in liquid N_2 and lyophilised. Analysis of ^{15}N content was outsourced to the Research Laboratory for Archaeology and the History of Art, University of Oxford, UK. Here, continuous flow isotope ratio mass spectrometry (CF-IRMS) on a Europa Scientific GEO20-20 machine was used to determine ^{15}N content.

2.8 Creation of TAP tag constructs

2.8.1 Amplifying gene targets by PCR

Two small leaves were taken from soil grown wild-type *Arabidopsis* and frozen in liquid N₂. RNA was extracted, DNase treated and reverse transcribed as described in 2.5.2, 2.5.3 and 2.5.4. Primers designed to amplify full-length CAT2, CAT6, CAT8 and LHT9 transcripts were ordered for N-terminal TAP tags, and primers for the same targets with the stop-codon removed for the C-terminal TAP tags were also ordered (Table 2.3). All forward primers contained an additional CACC at the 5' end for directional cloning in to pENTR-DTOPO (Life Technologies, Loughborough, UK).

Cloning PCRs were performed in 50 µl volumes containing 1-4 µl wild-type cDNA template, 1x Phusion high-fidelity reaction buffer (Life Technologies, Loughborough, UK), 1 µl 10 µM forward and reverse primers and 1 U Phusion high-fidelity DNA polymerase (Life Technologies, Loughborough, UK). Annealing times for each primer pair (listed in Table 2.3) were held for 30 seconds, followed by an extension period at 72°C for 1 minute kb⁻¹. A total of 30 annealing and extension cycles were used.

PCR products were visualised on an agarose gel, then excised and purified using GenElute gel extraction kit (Sigma-aldrich, Dorset, UK).

2.8.2 TOPO cloning of PCR cloning in to pENTR-DTOPO

CAT2, CAT6, CAT8 and LHT9 purified PCR products were separately inserted in to the pENTR-DTOPO entry vector. 3 µl of fresh PCR product was added to 1 µl TOPO cloning salt solution, 1 µl TOPO vector and 1 µl dH₂O then the reaction was incubated at room temperature for 5 minutes. One Shot TOP10 chemically competent *E.coli* (Life Technologies, Loughborough, UK) were transformed with the reaction product using the heat-shock method and then spread on pre-warmed 50 µg ml⁻¹ kanamycin LB plates.

Individual colonies were isolated and cultured in 50 $\mu\text{g ml}^{-1}$ kanamycin liquid LB overnight. Plasmid DNA was extracted from these cultures using GenElute plasmid miniprep kit (Sigma-Aldrich, Dorset, UK) and following the manufacturer's instructions. PCR using target specific primers was used to check that the PCR product had inserted in to the entry vector.

2.8.3 Transfer of transgenes to Gateway-ready TAP tag binary plasmids

Transgene inserts were transferred from pENTR-DTOPO vectors to binary vectors containing C- and N-terminal TAP tags, ctapi.289.gw and ntapi.289.gw (Rohila et al., 2004), respectively. Transgenes missing stop codons were transferred to ctapi.289.gw and transgenes with stop codons were transferred to ntapi.289.gw. The LR recombination reaction was used to transfer transgenes, which used 2 μl LR Clonase II (Life Technologies, Loughborough, UK), 150 ng transgene containing plasmid DNA, 150 ng destination vector and 6 μl TE buffer pH 8.0. Reactions were incubated at 25°C for one hour, then transformed in to TOP10 chemically competent cells and then spread on pre-warmed 100 $\mu\text{g ml}^{-1}$ spectinomycin LB plates.

Individual colonies were tested by PCR for the transgene as described above before sending for sequencing. All sequencing was carried out by Source Bioscience, UK. Transgene sequences were checked for alterations against the original sequence as a reference using CodonCode Aligner software. Only unaltered, in-frame constructs were allowed to pass beyond this point.

2.8.4 Chemically competent *Agrobacterium tumefaciens* GV3101 preparation

Agrobacterium tumefaciens GV3101 was spread on LB agar containing 25 $\mu\text{g ml}^{-1}$ gentamycin and grown overnight at 28°C. A single colony was picked and grown overnight in 5 ml LB containing 25 $\mu\text{g ml}^{-1}$ gentamycin at 28°C with 170 rpm shaking. This culture was used to inoculate 100 ml LB containing 25 $\mu\text{g ml}^{-1}$ gentamycin and was grown at 28°C with 170 rpm shaking until an OD₆₈₀ of 0.6-1.0 was achieved. The culture was placed on ice and

then centrifuged at 1,750g for 6 min at 4°C. The supernatant was discarded and the pellet was gently resuspended by pipetting in 1.4 ml of ice-cold filter-sterilised 20 mM CaCl₂. 200 µl aliquots were frozen on liquid nitrogen and stored at -80°C.

2.8.5 Agrobacterium transformation

Competent agrobacteria were thawed on ice with 2 µl (0.5-2 µg) transgene containing purified plasmid for 30 minutes. Cells were then frozen in liquid nitrogen followed by incubation at 37°C for 5 minutes. 1 ml LB was added to the suspension by gentle pipetting. Incubation at 28°C with 150 rpm shaking for 4 h followed. Cells were then centrifuged at 16,000 xg for 30 seconds. Pellets were resuspended in 100 µl LB and spread onto LB agar containing 25 µg ml⁻¹ gentamycin and 100 µg ml⁻¹ streptomycin. Plates were sealed with Parafilm and incubated for two to three days at 28°C.

2.8.6 Agrobacterium-mediated transformation of Arabidopsis using the floral dip method

Transformation was performed as described by Zhang *et al.* (2006). Agrobacterium cultures harbouring the gene of interest were grown in LB + antibiotic selection at 28°C for 2 days. This culture was then used to inoculate 500 ml LB with antibiotic selection and this larger culture was grown for 16-24 hours at 28°C, until growth entered the stationary phase (OD ~1.5 – 2.0). *Agrobacterium* cells were centrifuged at 4000 xg for 10 minutes at room temperature and resuspended in 300 ml of 5% (w/v) sucrose and 0.02% (v/v) Silwet L-77. Bolting Arabidopsis plants with siliques removed were then inverted and dipped in to the *Agrobacterium* for 20 seconds with gentle agitation. Excess liquid was allowed to drain off the leaves then plants were covered with plastic and left on their sides away from direct sunlight for 24 hours. Dipping was repeated twice more to ensure transformation was successful. Siliques were not removed after the first dipping. When plants reached maturity, seed was collected and positive transformants were selected by sowing on to basta selection plates.

2.8.7 Using Western Blot to select lines strongly expressing TAP-tag

T₀ seed transformed with 35S:CTAG-CAT2 and 35S:CAT2-NTAG were selected for transgene expression on Basta plates and positive transformants were transplanted to soil after 11 days. Lines were numbered and seed from individual plants was pooled together. Pooled T₁ seed were again selected for transgene expression on Basta plates. After 11 days, positive transformants were transplanted to soil and grown for a further 11 days before sampling ~10 g of leaf tissue. Leaf tissue was homogenised in liquid nitrogen using a pestle and mortar and 15 ml of protein extraction buffer (2% (w/v) sodium dodecyl sulfate (SDS), 100 mM Tris, pH 6.8, 10% (v/v) glycerol, 0.1% Triton-X, 5 mM EDTA, pH 6.8, 10 mM DLdithiothreitol (DTT) and 1 mM phenylmethanesulfonylfluoride (PMSF)) was added. Protein extracts were incubated at 4°C for 30 minutes then centrifuged at 10,000 xg for 15 min at 4°C to remove nuclei and cell debris. The supernatant was centrifuged at 80,000 xg for 40 min at 4°C to isolate membranes. Membrane suspension protein concentrations were quantified using a Bradford assay and equal amounts of protein were loaded on to a 12% SDS-PAGE gel. After the protein had successfully migrated through the gel, the gel was incubated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol), and protein was transferred to a nitrocellulose membrane using the Biometra B33-34 system running at 120 V for 60 min. The nitrocellulose membrane was blocked for 1 hour at 4°C with 5% (w/v) milk in 1x TBS-T buffer (1x TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), 0.1% (v/v) Tween20), with gentle agitation. Blocking solution was removed and the membrane was incubated with 1:5,000 peroxidase anti-peroxidase (PAP) antibody (which binds protein A in the TAP tag) in 5% (w/v) milk-TBS-T for 1 hour at 4°C. The membrane was washed 4 x 20 min in TBS-T to remove unbound antibody. Enhanced chemiluminescent (ECL) substrate (ThermoFisher, UK), which is a substrate for horseradish peroxidase (HRP), was incubated with the membrane for 5 min. Chemiluminescence was detected using ImageQuant LAS 4000 (GE Healthcare Life Sciences, UK).

2.9 Tandem Affinity Purification

2.9.1 Protein crosslinking and tonoplast isolation

Tap lines: 35S:CAT2-CTAG and 35S:NTAG-CAT2, plus knockout line *cat2-1* were germinated on ½ MS plates then transplanted into sand as described in section 2.2. Sand plants were all fed N-free Hoagland's + 1 mM NH₄NO₃. Sand grown, whole seedlings were harvested at 24 days after germination and incubated in ice-cold 1% formaldehyde in PBS buffer for 30 min (Bettey and Smith, 1993) to cross-link *in vivo* protein interactions. The reaction was quenched by adding ice-cold 300 mM glycine for 30 mins. Cross linked tissue was then washed with ice-cold PBS and plant material (~60 g fresh weight) was homogenised using a large pestle and mortar. 250 ml of a protein extraction buffer comprised of 1% (w/v) n-dodecyl d-maltoside (DDM), 100 mM 100 mM Tris (tris(hydroxymethyl)aminomethane) buffered to pH 6.8 with hydrochloric acid, 10% (v/v) glycerol, 0.1% (v/v) Triton-X, 5 mM EDTA pH 8, 10 mM DLdithiothreitol (DTT) and 1 mM phenylmethanesulfonylfluoride (PMSF). The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at 13 000 × g, for 15 min at 4°C. The resulting supernatant was centrifuged at 80000 X g (40 min, 4°C), and the pellet resuspended in a glycerol-containing medium based on that used by Pope and Leigh comprised of 1.1 M glycerol, 1.0 mM EDTA, 10 mM Tris and 2.0 mM DTT.

2.9.2 TAP tag purification

The tonoplast membrane suspension was centrifuged at 80000 x g (20 min, 4°C) and the supernatant discarded. The tonoplast pellet was resuspended in 2 ml solubilisation buffer, which consisted of 1% (w/v) DDM, 40 mM Tris-HCL pH 8.0, 300 mM NaCl, 2 mM EDTA, 20% (v/v) glycerol and 1 mM PMSF and transferred to a 2 ml Eppendorf tubes. Tonoplasts were left to solubilise by rotating for 30 minutes at 4°C. Whilst tonoplasts were solubilising, 160 µl Mouse IgG-Agarose beads (Sigma, Dorset, UK) were pipetted into Micro Bio-Spin

chromatography columns (Bio-Rad, Hertfordshire, UK). The foot of the column was removed and inserted into 2 ml eppendorf tube, then centrifuged at 900 rpm for 1 minute at 4°C. Flow-through was discarded. IgG beads were washed twice with 500 µl tris buffered saline (TBS) which consisted of 10 mM Tris-HCL pH 7.5 and 150 mM NaCl. Beads were then washed three times with 500 µl acetate buffer (3% (v/v) acetic acid and 0.5 M ammonium acetate, pH 3.4), followed by 4 times with TBS. The pH of the final flow-through was tested using pH test paper (Life Technologies, Loughborough, UK) to ensure the column had reached a level between 7.5 and 8.0.

Solubilised tonoplasts were centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant was transferred in to a fresh tube and a 100 µl sample was taken as a total solubilised sample. Using a cut pipette tip, IgG beads were transferred from the column to the tube containing solubilised tonoplasts. Tubes were sealed and left to rotate for 150 minutes at 4°C. At the end of the rotation step, tubes were centrifuges at 2,500 rpm for 2 minutes at 4°C. 100 µl of supernatant was removed as the post binding lysate. All but 700 µl supernatant was carefully discarded and beads were resuspended and transferred to the column. The column was washed with the foot off 7 times with 500 µl wash buffer (0.3% (w/v) DDM, 40 mM Tris-HCL pH 8.0, 300 mM NaCl, 2 mM EDTA, 20% (v/v) glycerol and 1 mM PMSF), centrifuging at 900 rpm for 1 minute at 4°C between washes. After the final wash, the column foot was replaced and 207 µl wash buffer, 3 µl His-TEV (Sigma, Dorset, UK) and 90 µl 0.5 M EDTA was added to the column. Columns were sealed well and rotated overnight at 4°C.

60 µl Ni-NTA slurry (Qiagen, Manchester, UK) was pipetted using a cut tip into a fresh column and centrifuged at 10,000 rpm for 1 minute at room temperature. The supernatant was discarded and beads were resuspended in 150 µl wash buffer. Equilibrated Ni-NTA slurry was added to the TEV column and rotated for 1 hour at 4°C.

Following the incubation, the foot and lid of the column was removed and columns were centrifuged at 1,500 rpm for 2 minutes at 4°C. The elution (~400 µl) was collected and kept on ice. 150 µl wash buffer and 150 µl 0.5 M EDTA were added to the column then the lid and foot replaced and columns were rotated at 4°C for 1 hour.

After one hour, the column foot and lid were removed and columns were centrifuged at 1,500 rpm for 2 minutes at 4°C. Elution was collected (~300 µl) and combined with the previous elution sample.

Calmodulin beads (GE Healthcare, Buckinghamshire, UK) were prepared by centrifuging 150 µl of beads and removing the supernatant. Beads were then resuspended in 150 µl binding buffer (50 mM Tris-HCL pH 7.5m 0.5 M NaCl and 2 mM CaCl₂). Calmodulin beads were added to the TEV elution sample and rotated for 1 hour at 4°C.

After one hour, tubes were centrifuged at 1,500 rpm for 1 minute at 4°C and the supernatant was removed. Beads were resuspended in 150 µl calmodulin elution buffer and transferred with a cut pipette tip to a fresh column. Columns were centrifuged at 1,500 for 2 minutes at 4°C with the foot off and the flow-through was collected (sample) and concentrated to ~75 µl using Millipore microcentrifuge tubes (Merck Millipore, Darmstadt, Germany).

2.9.3 Analysing protein-protein interaction

To determine whether CAT2 interacts with any other proteins, the products of the tandem affinity purification were run on a 12% SDS-PAGE gel. For each genotype (wild-type, *cat2-1*, 35S:NTAG-CAT2 and 35S:CAT2-CTAG) 3 samples were included; total solubilised protein (TS), post binding lysate (PBS) and final sample.

Gels were fixed in prefix buffer (50% (v/v) MeOH and 10% (v/v) HoAc) for 30 minutes then stained with prefix buffer + 0.25% Coomassie Blue R-250 for 4 hours. Gel was destained for

24 hours with 5% (v/v) MeOH and 7.5% (v/v) HoAc. Gels were scanned and stored for future reference in 7% (v/v) HoAc.

2.10 Measuring intracellular pyruvate using pyruvate oxidase assay

2.10.1 Sample preparation

Plant roots were sampled and frozen, liquid N₂ and ground to a fine powder using glass beads in the TissueLyser (Qiagen). An equal volume of ice cold 0.5 M perchloric acid was added and left to incubate for 5 minutes. Samples were centrifuged at 3000 xg for 2 minutes at 4°C. The acid was then neutralized by adding 50 µl of 5 M potassium carbonate. Precipitated potassium perchlorate and proteins were removed by filtration with a 0.2 µm PTFE syringe filter and filtered extract was centrifuged at 10,000 xg for 5 minutes. Supernatant was transferred to a new tube and diluted 3-fold with assay buffer (100 mM potassium phosphate with 1.0 mM EDTA, pH 6.7) to stabilise pH.

2.10.2 Pyruvate fluorescence assay

The assay was performed as described in Zhu *et al.* 2010. A pyruvate calibration curve was established using 20 µl volumes of pyruvate standards at 0, 200, 400, 600, 800, 1000, 1500 and 2000 pmol/well. 20 µl of sample extract was added to the remaining wells of a black 96-well plate. 180 µl of assay cocktail (100 mM potassium phosphate with 1 mM EDTA, pH 6.7, 1 mM MgCl₂, 10 µM FAD, 0.2 mM thiamine pyrophosphate, 0.2 U/ml pyruvate oxidase, 50 µM amplex red (Life Technologies, Loughborough, UK) and 0.2 U/ml horse radish peroxidase) was added to each well then mixed thoroughly and left to incubate at room temperature for 30 minutes. Fluorescence was recorded at 590 nm with excitation at 535 nm using a plate reader. Measurements were taken from a blank sample to control for background fluorescence.

2.11 Microscale Thermophoresis

2.11.1 CAT2-GFP overexpression and purification in *saccharomyces cerevisiae*

Overexpression and purification of CAT2 was carried out as described in Drew *et al.* (2008). All cloning steps and yeast cell selection were carried out by Jo Parker, Department of Biochemistry, University of Oxford. CAT2 was cloned into a GFP-8His-containing vector by homologous recombination using CAT2 specific primers that included 5' overhangs (35 bp) that complemented the upstream and downstream sequences to either side of the restriction enzyme site in the GFP-8His vector. 5 µl of CAT2 PCR product and 3 µl of linearized vector were incubated with competent *S.cerevisiae* cells for 30 min at 30°C, then heat shocked for 25 min at 42°C. Cell suspension was plated onto selective medium and incubated at 30°C for 2-3 days.

Yeast colonies were screened for GFP expression by inoculating 10 ml URA medium (2.0 g l⁻¹ of yeast synthetic drop-out medium without Ura, 6.7 g l⁻¹ yeast nitrogen base without amino acids and either 2% glucose (for pre-culture) or 0.1% glucose (expression culture)) supplemented with 2% glucose with a yeast colony and incubating overnight in an orbital shaker at 280 rpm at 30°C. 10 µl of overnight culture was spotted onto a fresh URA plate, dried at room temperature and transferred to a 30°C incubator for 1-2 days. The original overnight cultures were diluted to 0.1% glucose and incubated in an orbital shaker at 30°C until cultures reached an OD₆₀₀ of 0.6 (~7 hours). Expression of membrane protein-GFP fusion was induced by adding 2% (w/v) galactose. Twenty-two hours later, induced cells were centrifuged at 3000 xg for 5 min and the supernatant was discarded. Cells were resuspended in 200 µl YSB (50 mM Tris-HCl, PH 7.6, 5 mM EDTA, 10% (v/v) glycerol, 1 x protease inhibitor cocktail) and transferred to a black Nunc 96-well optical bottom plate. GFP fluorescence emission at 512 nm by excitation at 488 nm was measured in a microplate spectrophotometer. Membrane protein overexpression levels were estimated from the yeast

whole-cell fluorescence using the method described in Drew *et al.* 2008. The yeast cells with the best GFP fluorescence were selected.

Large-scale membranes were isolated by Inoculating 10 ml of URA media with the selected yeast culture and incubating at 30°C overnight. The next day, the overnight culture was added to a 500 ml shaker flask containing 150 ml URA medium and 2% glucose. This was Incubated overnight in an orbital shaker at 280 rpm. set at 30°C. The culture was diluted to an OD₆₀₀ of 0.12 into 1 l of URA containing 0.1% glucose in a 2.5 l shaker flask. This was then incubated in an orbital shaker at 280 rpm set at 30°C. When OD₆₀₀ reached 0.6, GFP expression was induced with 2% galactose. Cells were harvested after 22 h of incubation by centrifugation at 4,000 xg at 4°C for 10 min. The supernatant was decanted and the cell pellet was resuspended in 25 ml of CRB (50 mM Tris-HCl, PH 7.6, 1 mM EDTA, 0.6 M sorbitol) per liter of original cell culture. Cells were broken using a heavy-duty cell disruptor. Cells were passed through four times at incremental pressures of 25, 30, 32 and 35 kpsi (~ 1.7–2.4 × 10³ atm) at 4–15°C. 100 µl of cells were removed and transferred into a 96-well plate and GFP fluorescence was measured as previously described. Unbroken cells and debris were removed by centrifugation at 10,000 xg at 4°C for 10 min. The membrane containing supernatant was collected and 100 µl was transferred into a 96-well plate to measure GFP fluorescence as previously described. Yeast cell breakage efficiency was calculated by comparing GFP fluorescence to that measured after passing cells through the heavy-duty disrupter. To collect the membranes, the supernatant was centrifuged at 150,000g at 4°C for 120 min. The supernatant was discarded and the pellet was resuspended to a final volume of 6 ml l⁻¹ of original cell culture with cold PBS (1.44 g Na₂HPO₄ (8.1 mM phosphate), 0.25 g KH₂PO₄ (1.9 mM phosphate), 8.00 g NaCl, 0.2 g KCl in 1000 ml H₂O; adjust pH to 7.4 using 1 M NaOH or 1 M HCl). 100 µl of the membrane suspension was transferred into a 96-well plate to measure GFP fluorescence as previously described. The amount of overexpressed membrane protein was calculated as described in Drew *et al.* 2008.

To purify the CAT2 protein from the membrane suspension, the membrane suspensions were diluted to a final protein concentration of 3 mg ml⁻¹ using EB (1x PBS, 10 mM imidazole, pH 8.0, 150 mM NaCl, 10% glycerol (v/v), 3x DDM) plus an additional 0.3% DDM. The membranes were incubated at 4°C for 1 h with mild agitation to allow solubilisation. The unsolubilised material was removed by centrifugation at 100,000 xg at 4°C for 45 min. The supernatant was transferred into an appropriately sized glass beaker and 100 µl of the detergent-solubilized membranes was transferred to a 96-well plate to measure GFP fluorescence as previously described. The amount of GFP fusion was calculated as described in Drew *et al.* 2008.

Ni-NTA slurry (Qiagen, Manchester, UK) was prepared by using 2 ml of slurry per 1 mg of GFP fusion to be purified and equilibrating with five column volumes of EB. Equilibrated Ni-NTA resin was then added to the detergent-solubilised membranes and a magnetic stirrer was used to mix the solution at 4°C for 2-3 h.

After the incubation, the membranes and slurry were poured into a glass Econo-Column (BioRad). 100 µl of the flowthrough was transferred into a 96-well plate and GFP fluorescence was measured as described as in Drew *et al.* 2008. The protein binding efficiency of the fusion to the Ni-NTA resin can be calculated as described in Drew *et al.* 2008. The column was washed with 10-column volumes of EB then 1 M imidazole was added to EB to a final concentration of 30 mM and the column was washed a further 35 times. To elute the protein, 1 M imidazole was added to EB to a final concentration of 250 mM and passed through the column 2-3 times. 100 µl of the eluate was transferred into a 96-well plate GFP fluorescence was measured as described in Drew *et al.* 2008. The amount of GFP fusion eluted was calculated as described in Drew *et al.* 2008.

The flowthrough was concentrated using a centrifugal concentrator to 0.5 ml and injected onto a Superdex 200 10/300 column pre-equilibrated with buffer used for dialysis at a flow rate of 0.5 ml min⁻¹. 0.5 ml fractions were collected and the UV-trace was used to collect and

pool the protein peak. The eluate (membrane protein-GFP fusion), the flowthrough (membrane protein before size exclusion chromatography (SEC)) and the protein peak (membrane protein after SEC) were analysed by SDS-PAGE.

2.11.2 Microscale thermophoresis binding assay

The amino acids to be tested were made up to 20 mM each and used in a serial dilution which ranged from 3 - 10 mM. The protein fraction containing the CAT2 fusion protein was diluted to 0.8 μ M and 10 μ l was added to each vial of amino acid. Amino acid and protein mixtures were drawn by capillary action through glass capillaries and placed into the Monolith NT.115 microscale thermophoresis machine (NanoTemper Technologies). The machine was operated according to manufacturer's instructions and data was analysed using the NT analysis software.

Chapter 3: The development of a growth-response screen to capture nitrogen-status sensing mutants in *Arabidopsis thaliana*.

3.1 Introduction

The aim of the work described in this chapter was to develop a robust growth system which could non-destructively quantify the growth of *Arabidopsis* plants whilst being able to precisely control the nutrient input. Further, the work aimed to establish whether the presence of exogenous amino acids generates a signal of nitrogen sufficiency and whether this leads to altered growth that could form the basis of a nitrogen-status sensing mutant screen.

3.1.1 Nitrogen-status sensing

It is widely accepted that plants have the capacity to sense nitrogen availability both externally in the soil and internally. Whilst there is evidence that the nitrate transporter NRT1.1 is involved in sensing external nitrate (Ho et al., 2009), we currently have limited understanding of how plants sense their internal nitrogen status. This chapter focuses on the development of a growth-response screen to identify mutants with altered nitrogen-status sensing.

3.1.2 Rationale for screen design

Some amino acids (Ala, Asn, Arg, Glu and Gln), when exogenously supplied to plant roots, inhibit the uptake of nitrate (Lee et al., 1992; Muller, 1992; Muller et al., 1995). The mechanism controlling this response is not currently understood, however it is likely to be the result of nitrogen-status signalling. The amino acids may be sensed internally as a signal of nitrogen sufficiency, which once sensed would feedback to regulate nitrate uptake in accordance with demand. We also know that the inhibition of nitrate uptake is accompanied by an inhibition of growth when amino acids are supplied (Behrend and Mateles, 1975;

Svennerstam et al., 2008), which could potentially be exploited in a simple phenotypic growth-response screen for nitrogen-status sensing mutants. Mutants with growth that is insensitive to exogenous amino acids are likely to be either perturbed in nitrogen-status sensing, or unable to internalise the supplied amino acid. To distinguish between these two scenarios, a second screen will need to be applied to any mutants identified. It is likely that the reduction in nitrate uptake is accompanied by changes in the abundance of transcripts relating to nitrate uptake and assimilation, which is an aspect that this chapter will aim to uncover. If changes in transcripts are detectable, this could be used as a second screening method to identify mutants that may be involved in nitrogen-status sensing.

3.1.3 Growth assay development

Many factors were considered when designing the growth assay. These included; the growth medium, the source of inorganic nitrogen, the selection of an appropriate concentration of inorganic nitrogen, the concentration of exogenous amino acid, the number of replicates to use in the screen and the experiment end point. Each of these points will be discussed in detail below.

3.1.4 Selecting the soilless growth medium

The first obvious factor to consider when designing the growth screen was the growth medium in which to grow the plants. The medium had to provide enough structure to allow the roots to grow and anchor the plant as well as be absorbent enough to retain nutrient solutions. The medium also had to be free of any nitrogen sources to enable complete experimental control of this essential nutrient. Previous attempts to precisely control nutrient input involved using various forms of media, including peat (Tschoep et al., 2009; Sulpice et al., 2013), sand (Chardon et al., 2010; Schellingen et al., 2014) and rockwool plugs (Bussell et al., 2013; Schellingen et al., 2014), all three of which were tested for suitability for the screen.

3.1.5 Non-destructive growth quantification

Traditional methods for quantifying the growth of plants involve measuring the dry weight of the roots and shoots. The disadvantage of this technique is that it can only be used once per plant due to the destructive nature of the method. Arguably a non-destructive method would be more beneficial, especially when conducting a reverse-genetic screen as this would allow the mutants to be analysed at multiple time points increasing the probability of capturing a growth phenotype. For this reason, growth was quantified by measuring leaf area by time-lapse imaging.

3.1.6 Nitrogen source selection

Nitrogen is an abundant element that is present in a variety of inorganic molecules that are readily available to plants. For instance, plants can take up and assimilate nitrogen in the form of urea, ammonium nitrate, potassium nitrate or ammonium sulfate and all of these sources are commonly found in many varieties of fertiliser and soil. The growth of *Arabidopsis* on high levels (>10 mM) of varying ratios of ammonium and nitrate was characterised by Hachiya et al., 2012. Growth has also been characterised on 5 mM ammonium nitrate (Cao, 1993), 2.5 mM ammonium nitrate (M'rah Helali et al., 2010), as well as 1-2.5 mM (Tsay et al., 1993). However, a comprehensive growth curve using extensive concentrations of ammonium nitrate has never been produced. Consequently, one of the aims of this chapter is to first characterise the growth response of *Arabidopsis* on a wide range of ammonium nitrate concentrations. Ammonium nitrate was selected as the nitrogen source because both nutrients are present abundantly in the soil (Balik et al., 2003; Sadej and Przekwas, 2008), and because growth has been shown to be enhanced, even surpassing maximal growth, when these nutrients are supplied together (Weissman 1964; Cox & Reisenauer 1973; Heberer & Below 1989; Hagin et al. 1990; Gill & Reisenauer 1993).

3.1.7 Variability of Arabidopsis growth

The growth of individual Arabidopsis plants is variable. This therefore ruled out the possibility of conducting a forward genetic screen in which growth of single mutant plants is the phenotypic readout. However, if the average growth of multiple plants of the same genetic line can be discriminated, a reverse genetic screen of candidate knockout lines would be a feasible option. For this to be possible, growth must be reliably and reproducibly consistent between batches of plants grown under the same conditions.

3.2 Results

3.2.1 A method for non-destructive quantification of vegetative growth

A non-destructive method for growth quantification was required so that multiple time points could be measured in order to increase the probability of capturing a growth phenotype during the screen. Measuring leaf area is a non-destructive alternative to measuring plant biomass as an output of growth. Arabidopsis produces a flat rosette during its vegetative growth phase, making it the ideal plant for leaf area quantification. An in-house programme written in Matlab, called Leaf lab, was developed by Dr Mark Fricker, which quantifies leaf area by measuring the area of the green leaf pixels (see Appendix B Figure 1 for an image of the program interface).

To determine whether quantifying leaf area was a good indicator of vegetative growth, leaf area was compared with vegetative dry weight. Aerial photographic images of plants at various stages of development were taken and leaf area was analysed using the Leaf lab programme. Once the images were taken, the plants were immediately dried and weighed. Leaf area and vegetative dry weight correlate well with an R^2 value of 0.9342 (Figure 3.1), which suggests that leaf area is reliable proxy for vegetative growth. However, the scatter of the data appears to increase with the size of the rosette. This is most likely caused by overlapping of leaves as the rosette increases in size, reducing the accuracy with which total leaf area is determined by the software.

This experiment also allowed us to decide the end time point for the screen. Images were taken every other day which enabled the plotting of a growth curve. The growth of Arabidopsis rosettes begins with a lag phase, followed by an exponential growth phase and ends with a stationary phase which is accompanied by bolting (Boyce et al., 2001). Day 23 after sowing was chosen as the screen end point as this represents the establishment of the exponential growth phase which, under the growth conditions used here, lasts from day 21

until day 27 after sowing. At this stage, seedlings are well established and large enough to reliably quantify rosette leaf area. This time-point also avoids any danger of the plants bolting, which occurs towards the end of the exponential growth phase at days 27-29.

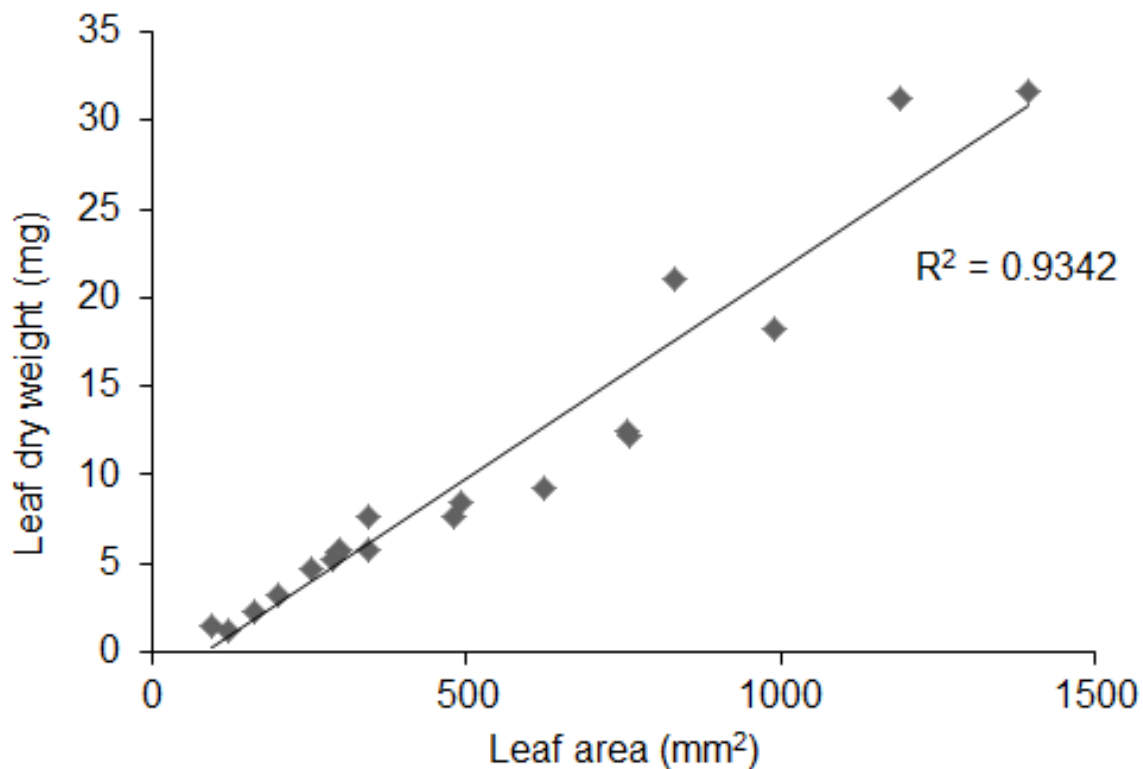


Figure 3.1. Correlation of leaf area with rosette dry mass.

Wild-type *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution + 0.5 mM NH_4NO_3 . Rosettes were photographed, sampled, dried and weighed every other day between 11 and 30 days after sowing. Leaf area was quantified using Leaf lab software.

3.2.2 Selection of soilless growth medium

Rockwool plugs, sand and peat were all tested as potential growth media. Seeds were sterilised and sown on to Agar plates containing ½ MS medium for 11 days to allow seedling establishment and uniform germination rates. Seedlings were subsequently transplanted to

the growth media to be tested. The peat was mixed with 30% (w/v) coarse vermiculite and 20% (w/v) fine vermiculite and supplemented with 260 mg K_2HPO_4 , 396 mg 396 mg GRANUKAL 85 (80% $CaCO_3$ and 5% $MgCO_3$ – Kreidewerke Dammann KG, Soehle, Germany) and 1.6 mg Fetrilon-Combi micronutrient fertiliser (BASFAG, Ludwigshafen, Germany) per 100 cm³ peat. Peat pots were watered with 0.5 mM NH_4NO_3 every other day. The sand and Rockwool plugs were watered with nitrogen-free Hoagland's solution plus 0.5 mM NH_4NO_3 . Rosette leaf area was recorded every other day (Figure 3.2). Plants fared the worst on Rockwool plugs. Plants died early on in the experiment due to algae and fungal infections on the moist plug surface and therefore leaf area data could not be collected.

Vegetative growth was fastest in the peat grown seedlings; however seedlings also grew well in the sand and vermiculite mixture (Figure 3.2). It is generally accepted that peats vary in the concentration of nitrogen containing compounds. One study suggests that a wide range of peats contained between 1.5 and 2.5% nitrogen (Lishtvan, and Korol; 1975), while another study suggested that sphagnum peat contains on average 1% nitrogen and other peats contain 1-4% nitrogen (Feustel, and Irvin; 1930). Combined, these studies confirm that peat contains variable amounts of nitrogen, which may interfere significantly with the ability to experimentally control the nitrogen input. Sand on the other hand, is completely nitrogen free. Based on this fact, sand was selected as the growth medium in order that nitrogen input could be precisely controlled.

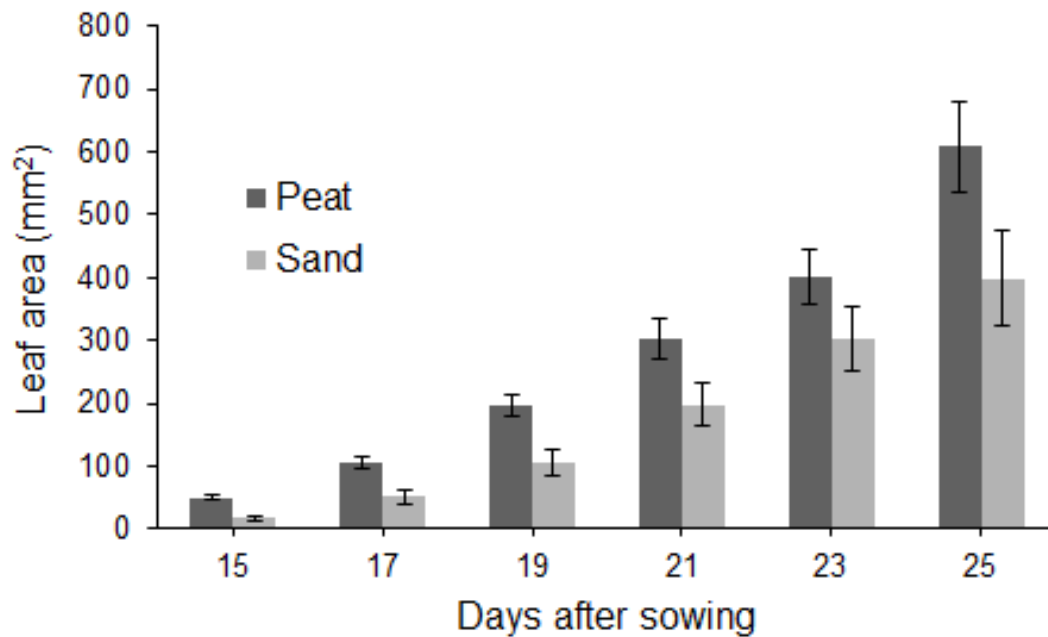


Figure 3.2. A comparison of the vegetative growth of Arabidopsis on sand and peat media.

Wild-type Arabidopsis seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were transplanted to either peat supplemented with N-free slow-release fertiliser or sand and vermiculite mixture. Seedlings on sand were watered every other day with N-free Hoagland's solution + 0.5 mM NH_4NO_3 . Seedlings on peat were watered every other day with 0.5 mM NH_4NO_3 . Plant images were taken every other day and leaf rosette area was quantified using Leaf lab software. Data are the mean of $n = 12 \pm \text{SEM}$.

3.2.3. Establishing wild-type rosette growth responses to varying concentrations of nitrogen

Nitrate and ammonium are two abundant nutrients in soil that can be readily taken up by plants. Because of this, these nutrients are commonly combined and fed to plants as ammonium nitrate under experimental conditions and in fertiliser applications. Although there are many examples in the literature as to what constitutes low and high ammonium nitrate concentrations, no detailed experiments have been published that characterise the growth response of Arabidopsis on a wide range of ammonium nitrate concentrations. Here I have

used concentrations of ammonium nitrate ranging from 0 – 10 mM to demonstrate that the vegetative growth of *Arabidopsis* has a biphasic response (Figure 3.3 A).

To investigate the biphasic growth response further, NH_4^+ and NO_3^- salts were fed individually in otherwise identical growth experiments (Figures 3.3 B and C). In contrast to the biphasic growth on ammonium nitrate, plants responded to $\text{Ca}(\text{NO}_3)_2$ in a monophasic manner with a plateau of growth beginning around 0.5 mM $\text{Ca}(\text{NO}_3)_2$ and evidence of growth inhibition at 10 mM $\text{Ca}(\text{NO}_3)_2$ (Figure 3.3 B). However, vegetative growth of seedlings fed increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ exhibited a similar biphasic pattern as seen in plants fed ammonium nitrate (Figure 3.3 C). Growth was greatest on the lower 0.1 and 0.5 mM concentrations and growth was most inhibited at 2 and 3 mM, with growth again increasing towards the higher 10 mM concentrations (Figure 3.3 C).

For all future experiments, 1 mM ammonium nitrate will be used as a nitrogen-limited concentration, and 4 mM will be used for high nitrate treatments.

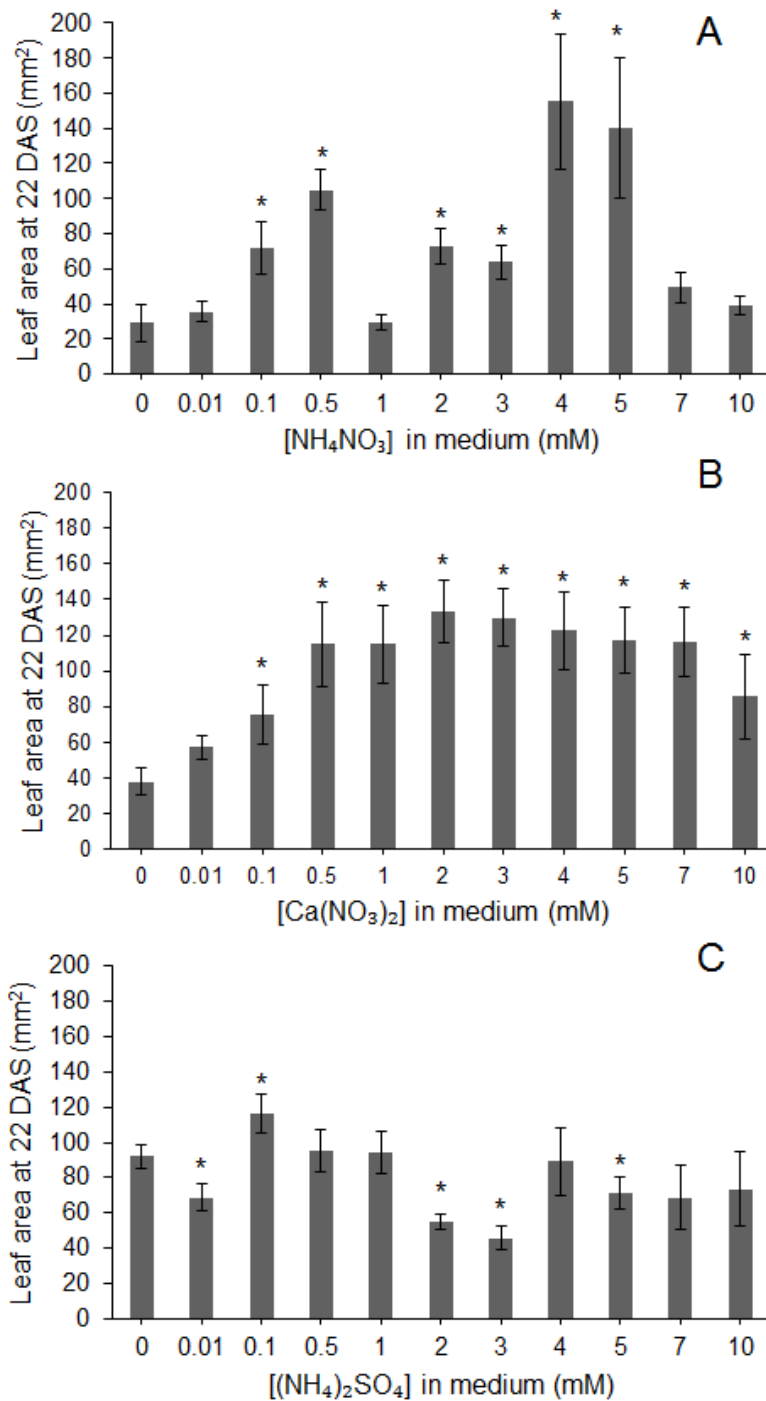


Figure 3.3. The effect of different concentrations of inorganic nitrogen on the vegetative growth of Arabidopsis.

Wild-type Arabidopsis seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus the indicated concentrations of NH₄NO₃ (A), Ca(NO₃)₂ (B) and (NH₄)₂SO₄ (C). Plants were photographed every other day and leaf area was quantified using Leaf lab software. Data are the mean of n = 12 ± SEM. Values significantly different from 0 control at p < 0.05 are indicated by * (t-tests).

3.2.4 Reliably detecting growth differences and evaluating growth variability

To conduct a successful reverse genetic growth-response screen, it must be possible to reliably detect growth differences between genotypes, and these differences must be reproducible between batches of plants. To verify this, I used a known slow growing mutant (*chl1-5*) and sowed this alongside wild-type (Figure 3.4). The results suggest that growth differences between mutant and wild-type can be reliably detected using this growth assay.

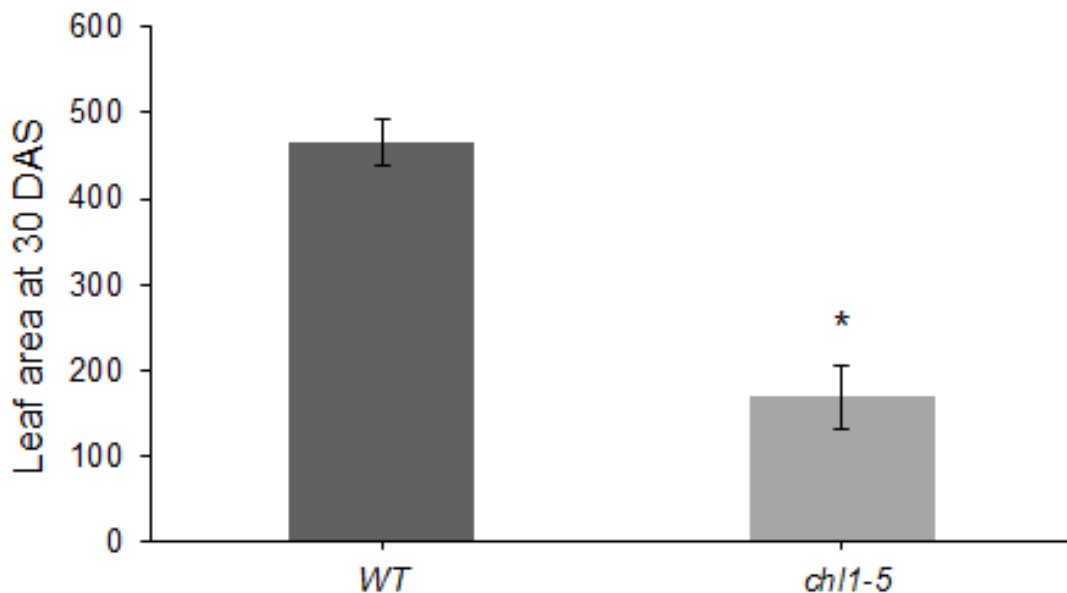


Figure 3.4. Using a slow growing mutant to test whether growth differences from wild-type can be reliably detected.

Wild-type and *chl1-5* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution + 1 mM NH_4NO_3 . Plants were photographed at 30 days after sowing and leaf area was quantified using Leaf lab. . Data are the mean of $n = 12 \pm \text{SEM}$. *chl1-5* was significantly different from wild type at $p < 0.05$ as indicated by * (t-test).

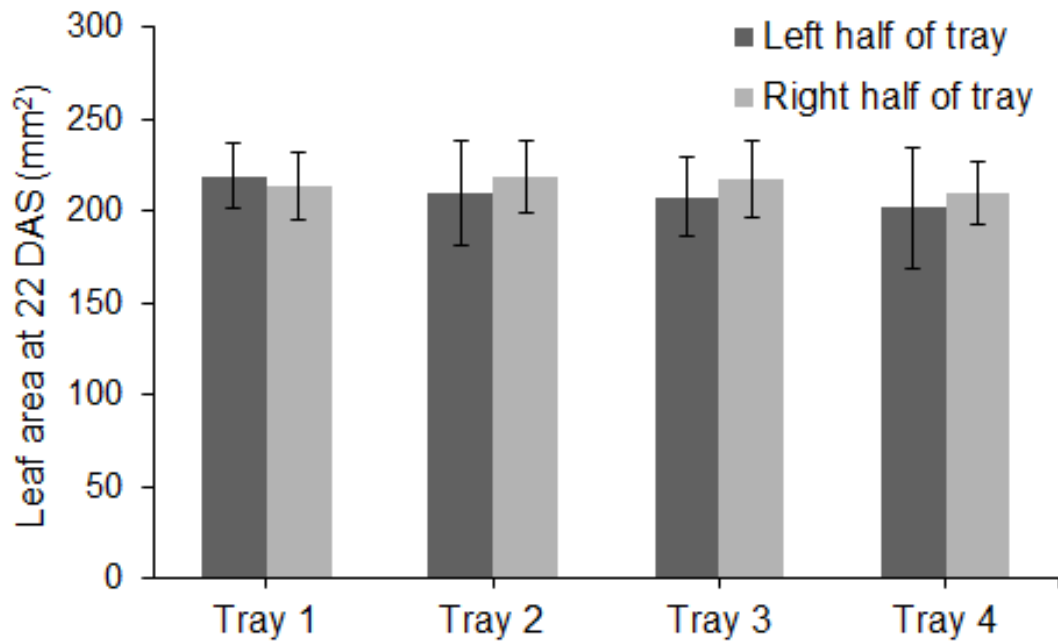


Figure 3.5. Testing the effect of position on the variability of *Arabidopsis* rosette growth.

Wild-type *Arabidopsis* seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution + 1 mM NH_4NO_3 . Trays of 24 cells were divided in to groups of 12 on either the left or right hand side to allow comparisons of the variability within trays as well as between trays. Plants were photographed every other day and leaf area was quantified using Leaf lab software. Data are the mean of $n = 12 \pm \text{SEM}$. ANOVA analysis showed no significant difference in leaf area either within or between trays at $p < 0.01$.

I was also interested to know whether growth under the same conditions could be reproduced between batches of plants in different trays, and within the same tray. Accordingly, I analysed multiple batches of wild-type plants, grown under the same conditions and compared the leaf area of plants within the same tray, and between trays (Figure 3.5). The average growth of *Arabidopsis* rosettes within a single tray, or between trays, does not differ significantly (Figure 3.5). These results suggest that the position within the growth chamber will not significantly impact on rosette growth, and that growth is reproducible between batches of plants.

Collectively, these results indicate that in using this growth assay, rosette size can be reliably and reproducibly used to discern growth differences between wild-type and mutant plants.

3.2.5. The effect of exogenous alanine on vegetative growth and nitrate uptake

Alanine, when exogenously supplied to plant roots, has been shown to inhibit the uptake of nitrate (Lee et al., 1992; Muller et al., 1995). The next logical question to ask was whether the inhibition of nitrate uptake affects the growth of the plant. A series of growth experiments were designed to investigate this. Firstly, alanine was supplied to Arabidopsis roots in increasing concentrations as the sole source of nitrogen (Figure 3.6). This was to determine the baseline growth response to alanine, as well as to confirm that Arabidopsis is able to take up this amino acid and metabolise it to successfully support growth.

Wild-type Arabidopsis appeared to grow particularly well on concentrations of alanine up to 1 mM (Figure 3.6). In fact, leaf area of plants grown at 1 mM alanine was greater than that of plants grown on 0.5 mM ammonium nitrate, and only 30 mm² less than the leaf area of plants grown on 4 mM ammonium nitrate (Figure 3.3 A). Above 1 mM, there is a dramatic decrease in growth which is maintained through to the higher 10 mM concentration. These results suggests that Arabidopsis can indeed take up and metabolise alanine as the sole nitrogen source but that higher concentrations of alanine are inhibitory to growth.

Next, we wanted to know whether supplying exogenous alanine led to an accumulation of alanine in the root and shoot tissues. An accumulation of alanine might generate a signal of nitrogen sufficiency, which in turn could cause the inhibition of nitrate uptake. To confirm this, I fed Arabidopsis plants a range of concentrations of alanine, and quantified the intracellular alanine concentration in shoots and roots (Figure 3.7).

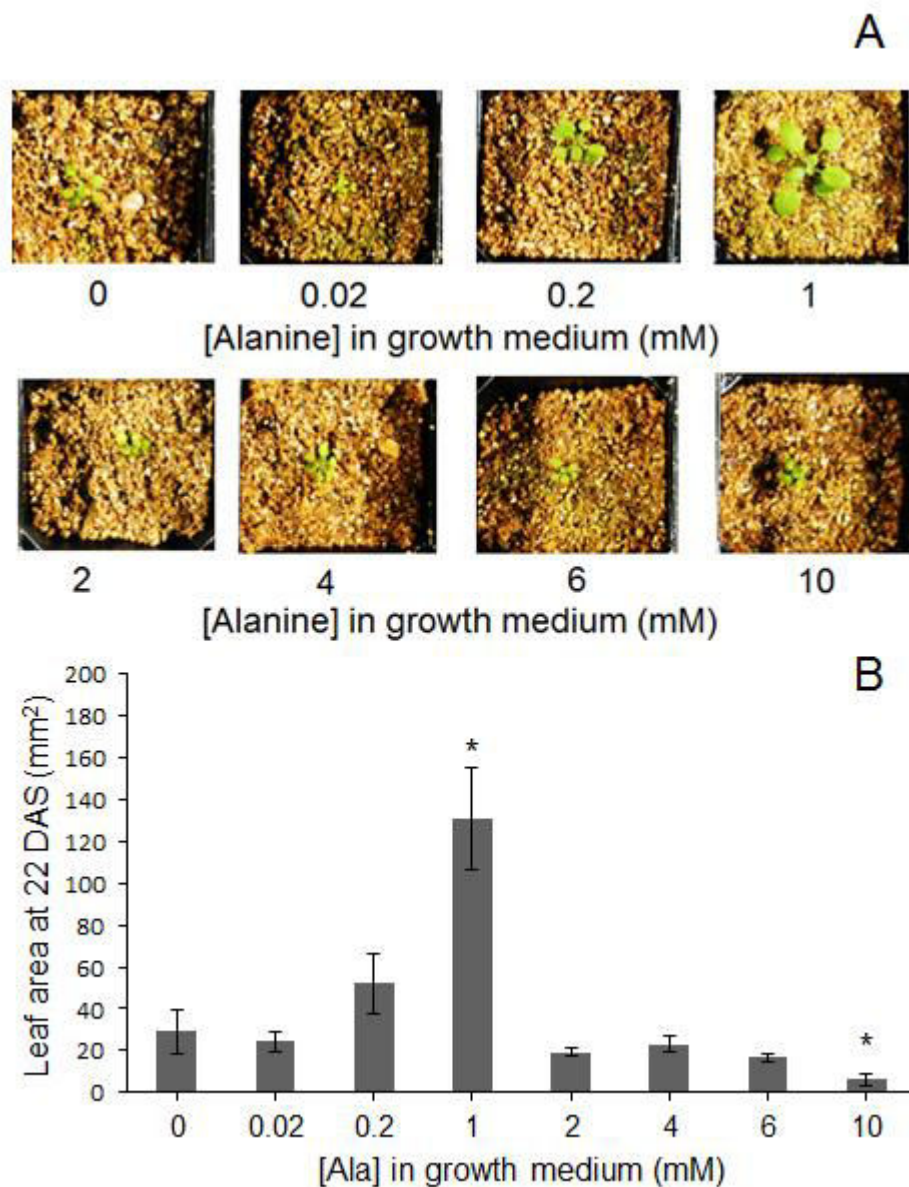


Figure 3.6. The effect of feeding alanine as the sole nitrogen source on the vegetative growth of *Arabidopsis*.

Wild-type *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus the indicated concentrations of alanine. Plants were photographed every other day and leaf area was quantified using Leaf lab software. Representative images of 22 day old plants (A), and quantified leaf area (B). Data are the mean of $n = 12 \pm \text{SEM}$. Values significantly different from 0 control at $p < 0.05$ are indicated by * (t-test).

Alanine accumulated to the greatest degree in the roots of plants that were fed 1 mM alanine (Figure 3.7 A). This is the same concentration at which vegetative growth was the greatest

when alanine was provided as the sole nitrogen source (Figure 3.6). These data provide evidence that *Arabidopsis* can survive on alanine as the sole nitrogen source, and that alanine accumulates in root tissues after alanine feeding. Further, supplying 1 mM alanine produces vegetative growth that is greater than that produced by concentrations of ammonium nitrate below 4 mM.

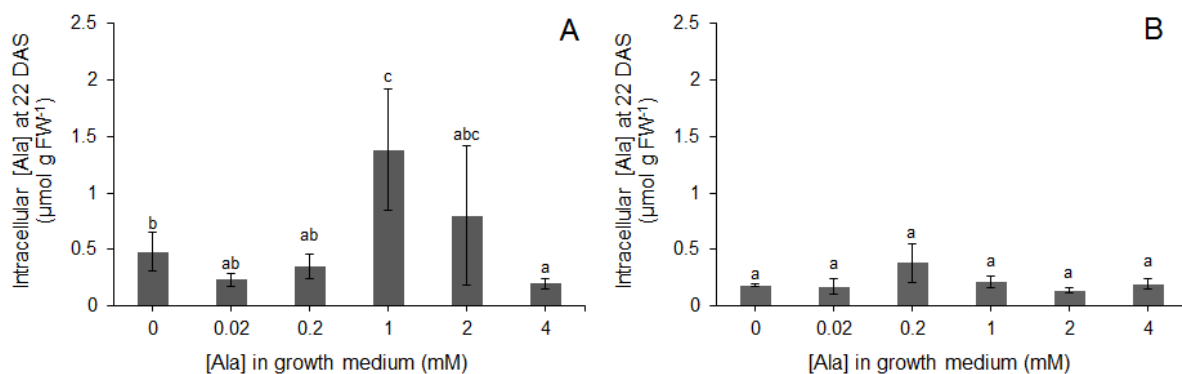


Figure 3.7. Alanine content in roots and shoots of plants fed exogenous alanine.

Wild-type *Arabidopsis* seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus the indicated concentrations of alanine. Roots (A) and shoots (B) were sampled at 22 days after sowing, 6 hours into the photoperiod. Data are the mean of $n = 6 \pm \text{SEM}$. Letters denote significance at $p < 0.05$ (t-tests).

In a separate experiment, exogenous alanine was fed to wild-type plants and GC-MS was used to quantify a number of other amino acids. This revealed that glutamate also appears to accumulate in the roots of *Arabidopsis* fed exogenous alanine (Figure 3.8). In the shoots of alanine fed plants, an increase in alanine was again observed, in contrast, however, a decrease in glycine, isoleucine, serine, glutamate and asparagine was observed (Figure 3.8). Such decreases in multiple shoot amino acids is surprising as one might expect that alanine treatment would have a positive effect on the nitrogen status of the plant, given the observed growth phenotype. Such a result indicates that nitrogen status signalling is complex. Perhaps the provision of exogenous alanine to the roots has perturbed nitrogen

status is such a manner that the plant is operating as though there is a greater availability of amino acids than is actually the case.

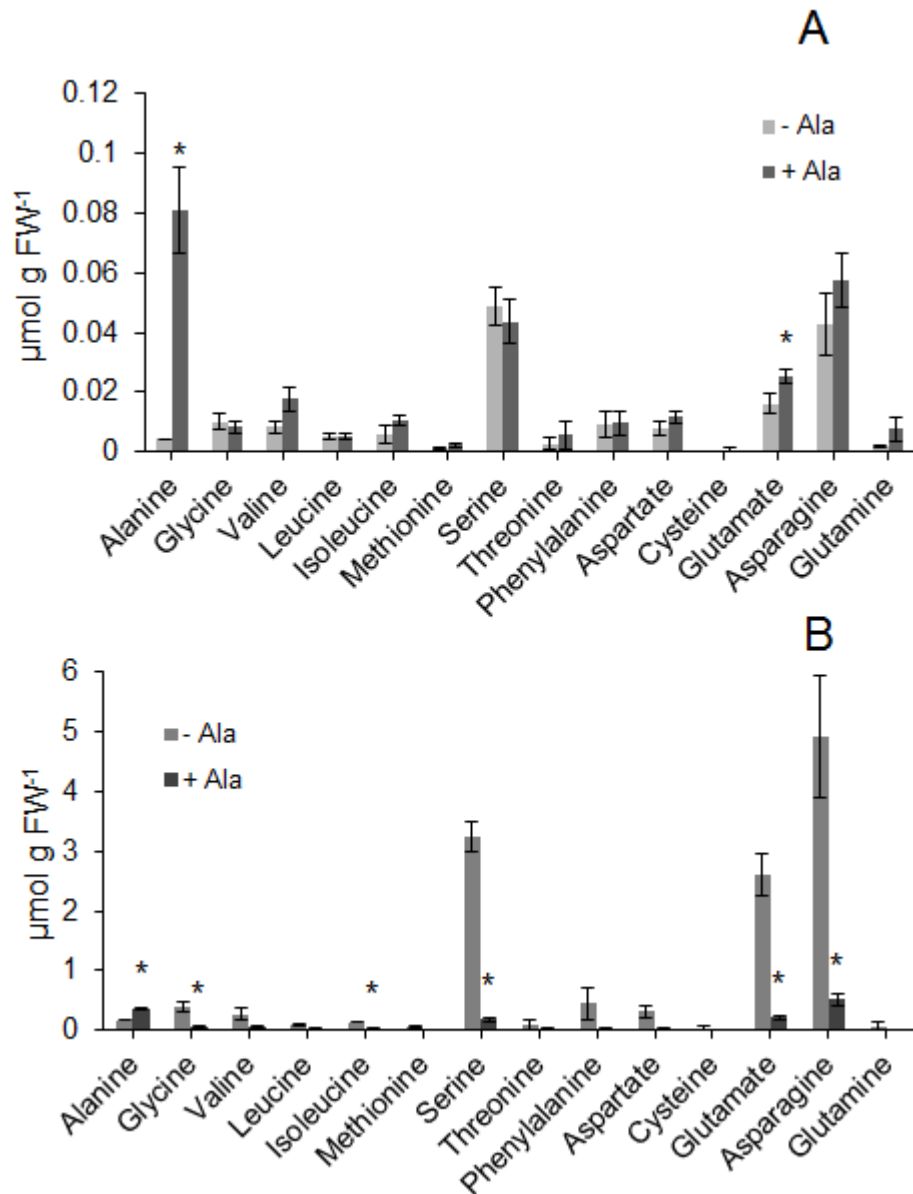


Figure 3.8. The effect of exogenous amino alanine on the concentration of other amino acids in the shoots and roots of *Arabidopsis*.

Wild-type *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus either 1 mM NH₄NO₃ or 1 mM NH₄NO₃ and 1 mM alanine. Roots (A) and shoots (B) were sampled at 22 days after sowing, at 6 hours in to the photoperiod. Data are the mean of $n = 6 \pm \text{SEM}$. Values significantly different from –Ala controls at $P < 0.05$ (t-test) are indicated by *.

A second growth experiment was set up to test whether the reported alanine induced inhibition of nitrate uptake would also affect vegetative growth. Seedlings were germinated and established on ½ MS plates and transplanted to sand 11 days after sowing. Seedlings were split into groups of 12 and were fed, nitrate, alanine or nitrate and alanine. Various combinations of the two nutrients were tested (Figure 3.10).

To characterise the nitrate uptake response in the presence of alanine in my growth assay, I fed wild-type plants 0.5 mM K¹⁵NO₃ + 0.5 mM (NH₄)₂SO₄ (for minus alanine treatment), or 0.5 mM K¹⁵NO₃ + 0.5 mM (NH₄)₂SO₄, and 1 mM alanine (for plus alanine treatment) (Figure 3.9).

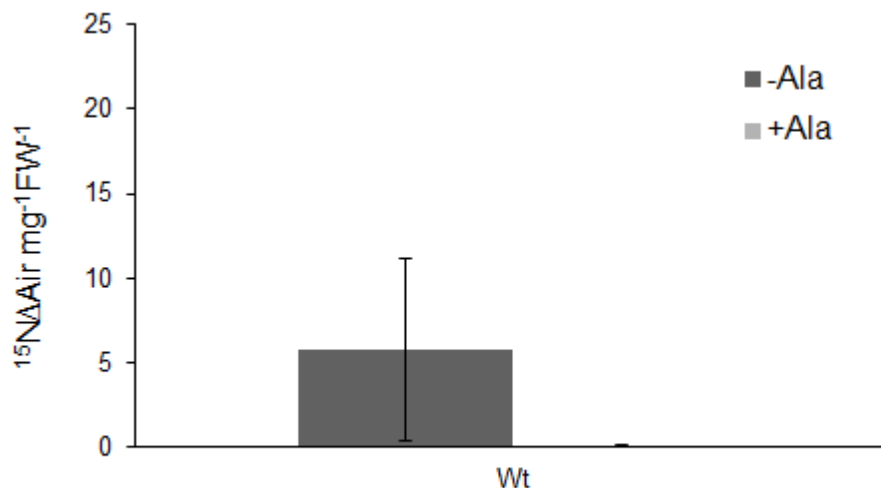


Figure 3.9. The effect of alanine on nitrate uptake.

Arabidopsis wild-type seeds were sterilised and sown on to plates containing ½ MS. After 11 days, seedlings were transplanted to flasks containing 0.1 mM CaCl₂ for a 24 hr starvation period. After 24 hours, wild-type seedlings were fed either 0.5 mM K¹⁵NO₃ + 0.5 mM (NH₄)₂SO₄ (-Ala), or 0.5 mM K¹⁵NO₃ + 0.5 mM (NH₄)₂SO₄, and 1 mM alanine (+Ala). Seedlings were sampled after 5 minutes, freeze-dried, and the quantity of ¹⁵N was analysed using isotope-ratio mass spectrometry. Data are the mean of n=3 ± SEM. T-tests were performed on –Ala vs +Ala but data was not significant.

The scale on the y axis represents the amount of ¹⁵N detected relative to the amount of ¹⁵N in air and air was used to normalise the samples. The data obtained for wild-type is

consistent with other published data that nitrate uptake is inhibited when amino acids are exogenously supplied (Lee et al., 1992; Muller, 1992; Muller et al., 1995) (Figure 3.9). However, the data in this case was not significantly different due to large variations in ^{15}N detection in the –Ala samples.

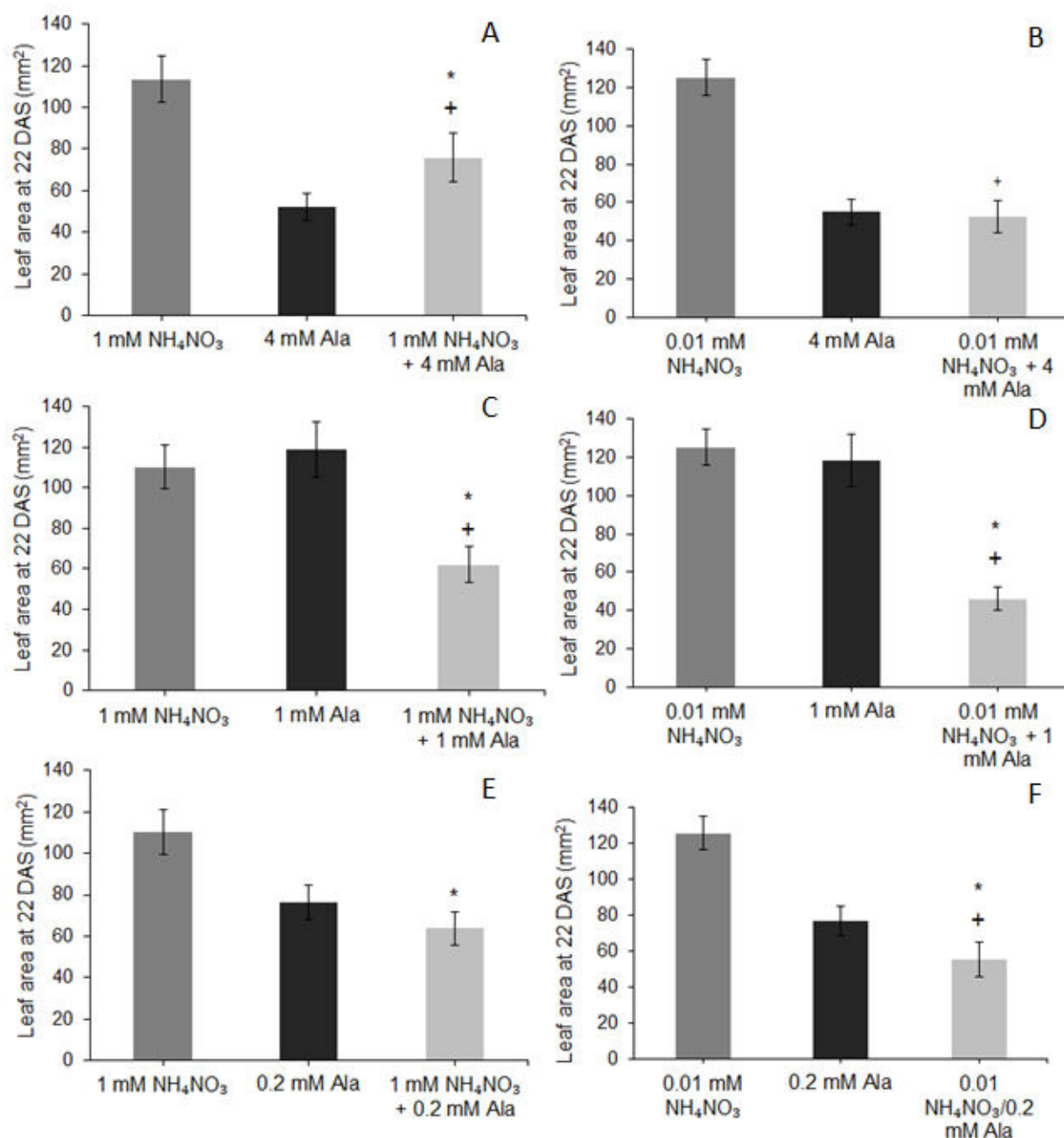


Figure 3.10. The effect of supplying a combination of alanine and NH₄NO₃ on the vegetative growth of Arabidopsis.

Wild-type Arabidopsis seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus the indicated concentrations of alanine and NH₄NO₃ (A – F). Plants were photographed every other day and leaf area was quantified using Leaf lab software. Data are the mean of $n = 12 \pm \text{SEM}$. Values significantly different from NH₄NO₃ at $p < 0.05$ are indicated by +. Values significantly different from alanine at $p < 0.05$ are indicated by *.

At all concentrations tested, when alanine and ammonium nitrate were supplied together, vegetative growth was significantly inhibited in comparison to vegetative growth on NH_4NO_3 alone (Figure 3.10 A - F). When 1 mM alanine was provided to plant roots, vegetative growth was equal to that of plants provided with 1 or 0.01 mM ammonium nitrate (figure 3.10 C, D), yet, when these concentrations of nutrients were supplied together, vegetative growth is inhibited.

The intriguing effects of alanine on growth stimulated the question of whether other amino acids, which have similarly been shown to inhibit nitrate uptake, also have an effect on vegetative growth. We selected the two pivotal amino acids, glutamine and glutamate and supplied them exogenously at a high concentration of 4 mM (Figure 3.11). The higher concentration was carefully chosen as this produced the largest growth inhibitory effect with alanine.

Both glutamine and glutamate significantly inhibited vegetative growth in comparison to the NH_4NO_3 control (Figure 3.11). Glutamine produced the same level of growth inhibition as alanine, with glutamate producing a slightly less inhibitory response than either glutamine or alanine.

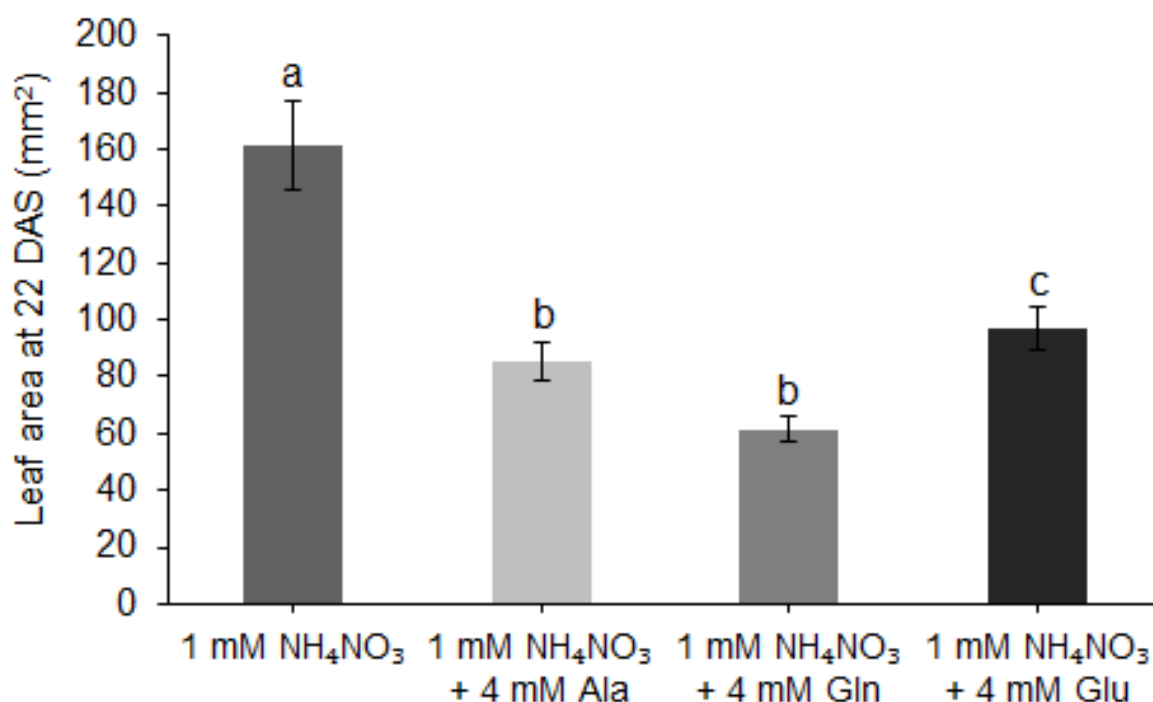


Figure 3.11. The effect of supplying a combination of three amino acids and NH_4NO_3 on the vegetative growth of Arabidopsis.

Wild-type Arabidopsis seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus the indicated concentrations of amino acid and NH_4NO_3 . Plants were photographed every other day and leaf area was quantified using Leaf lab software. Data are the mean of $n = 12 \pm \text{SEM}$. Letters denote significance at $p < 0.05$ (t-test).

3.2.6. Exogenous alanine reduces the abundance of nitrate uptake and assimilatory transcripts

To investigate whether the supply of exogenous alanine led to a reduction in the abundance of transcripts encoding the nitrate uptake and assimilatory machinery transcripts were measured in response to exogenously supplied alanine (Figure 3.12). 11 day old seedlings were starved for 24 hours prior to the addition of ammonium nitrate and alanine. This starvation period was to ensure that any residual nitrogen was removed from the roots, and to ensure that any effects of added ammonium nitrate and alanine on transcript abundance could be detected. Plant roots were sampled 24 hours after feeding for qRT-PCR analysis in order to determine the effect of alanine on transcript abundance. The abundance of the

transcript for the dual affinity nitrate transceptor, *NRT1.1*, is significantly reduced in response to the alanine treatment (Figure 3.12). Transcript abundances for the high affinity nitrate transporter *NRT2.2* and NADH-dependent glutamate synthase (*GLT1*) are also significantly reduced. No significant differences were observed in any of the other transcripts measured.

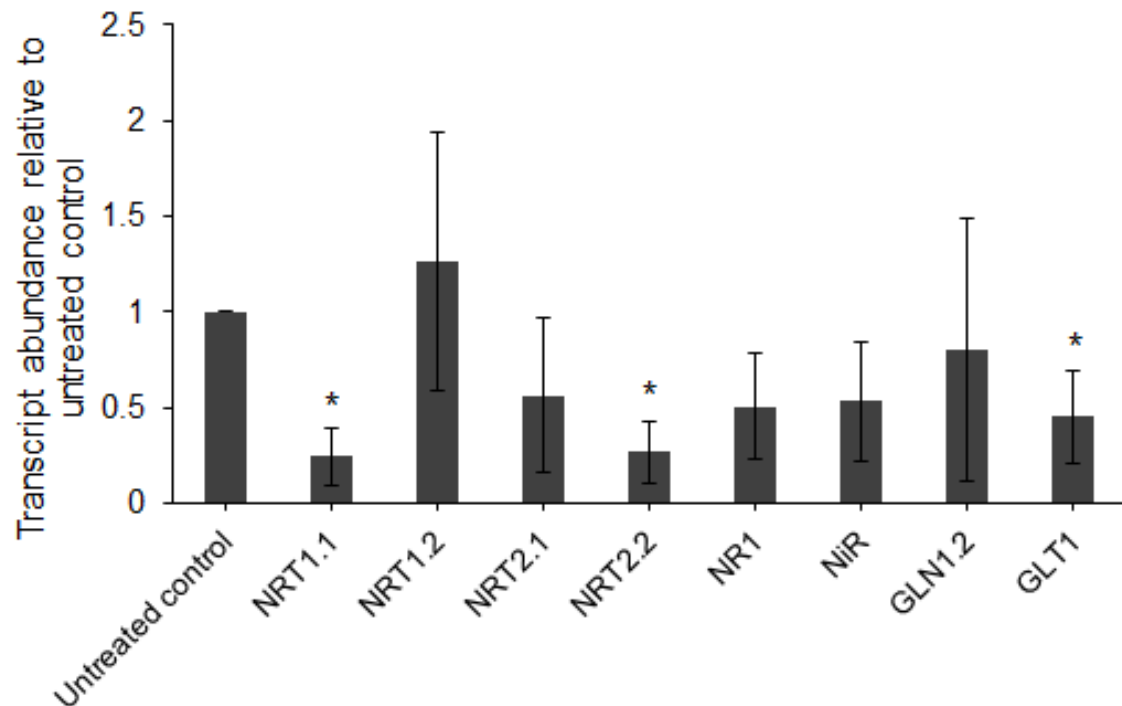


Figure 3.12. The effect of exogenously supplied alanine on nitrate uptake and assimilatory transcripts in wild-type Arabidopsis.

Wild-type Arabidopsis seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were starved for 24 hours in flasks containing 0.1 mM CaCl_2 . After 24 hours, 1 mM NH_4NO_3 + 1 mM Ala was added for a further 24 hours. Roots were sampled exactly 24 hours later for RNA extraction. RNA was transcribed to cDNA for qRT-PCR analysis. The transcript abundance was normalised to the housekeeping transcript *TIP41* and expressed relative to the untreated control (1 mM NH_4NO_3). Data are the mean of $n = 3 \pm \text{SEM}$. Values significantly different from the untreated control at $p < 0.1$ are indicated by * (t-tests).

3.3 Discussion

3.3.1 Arabidopsis has a biphasic growth-response to increasing concentrations of exogenous ammonium nitrate

Supplying increasing concentrations of exogenous ammonium nitrate to Arabidopsis roots results in a biphasic growth-response (Figure 3.3 A). One possible explanation for this biphasic response is that at lower concentrations of ammonium nitrate (0.01 – 0.5 mM) plants will preferentially take up ammonium rather than nitrate (Gazzarrini et al., 1999). The high affinity ammonium transporter 1.1 (AMT1.1) has a K_m of 0.5 μ M for its substrate ammonium (von Wiren et al., 2000), whereas the high affinity nitrate transport system has a K_m of 50 μ M for its substrate nitrate (Little et al., 2005). The kinetics of the two transport system are in support of ammonium being preferentially taken up at lower concentrations and thus the first growth peak is probably mainly the result of high affinity ammonium uptake. At higher concentrations (> 1 mM), ammonium uptake is possibly markedly reduced because of inhibition of AMT1.1 (Lanquar et al., 2009), therefore at concentrations above 1 mM it is likely that nitrate is preferentially taken up, contributing to the larger, latter growth peak.

When nitrate and ammonium salts are fed separately to plant roots, nitrate produces a monophasic response (Figure 3.3 B), whereas ammonium produces a similar biphasic response to that seen when ammonium nitrate is fed (Figure 3.3 A, C). This suggests that it is the presence of the ammonium that leads to the biphasic response observed when both nutrients are supplied together in an equal ratio. Seedlings supplied with 0.1 and 0.5 mM $(\text{NH}_4)_2\text{SO}_4$ displayed the highest growth, which supports the hypothesis that when ammonium and nitrate are co-provided, the first peak of growth is preferentially supported by high affinity ammonium uptake and that the latter, larger peak of growth in Figure 3.3 A is perhaps supported mostly by nitrate uptake. In support of this, there are evidence to suggest that when ammonium and nitrate are supplied together, the rate of nitrate efflux is greatly

reduced (Kronzucker et al., 1999), which could be another contributing factor to the second growth peak. Further to this, there is also evidence to suggest that ammonium uptake can be moderately stimulated by nitrate (Rideout et al. 1994; Saravitz et al. 1994; Kronzucker et al. 1999), which again could be a contributing factor to the latter, larger growth peak.

Ammonium has been shown to be toxic to most plants when fed in high concentrations as the sole source of nitrogen (Gerendás et al., 1997; Gerendas and Ratcliffe, 2000). It has been demonstrated that when the external concentration of ammonium is high, futile cycling occurs (Britto et al., 2001; Kronzucker et al., 2001; Masakapalli et al., 2013). Futile cycling arises when the efflux of ammonium is nearly equal to the influx of ammonium. This results in significant energy costs to the plant due to the active transport of ammonium across the membrane, and is accompanied by an overall reduction in ammonium uptake. Thus the result is a nitrogen deficient plant, expending significantly more ATP than under low ammonium/non-ammonium conditions, which displays symptoms of stress such as anthocyanin accumulation and growth stunting. Ammonium toxicity may contribute to the biphasic growth-response seen when feeding ammonium nitrate (figure 3.3 A) and ammonium sulphate (Figure 3.3 C). However, further experiments to measure ammonium influx and efflux would be required to determine this.

3.3.2 Exogenous alanine inhibits vegetative growth

Alanine, when exogenously supplied to plant roots in combination with ammonium nitrate, inhibits the vegetative growth of *Arabidopsis* (Figures 3.10 A - F). When supplied alone, alanine is taken up and is able to support growth, particularly at concentrations below 1 mM (Figure 3.6). However, when ammonium nitrate and alanine are supplied together, growth is always inhibited in comparison to growth on ammonium nitrate alone (Figure 3.10 A – F). This is consistent with data previously obtained that demonstrates the inhibition of nitrate uptake when amino acids are supplied exogenously (Lee et al., 1992; Muller et al., 1995). Growth is likely to be inhibited because of the inhibitory effect that alanine has on nitrate

uptake. Consequently, this results in a plant that has an overall reduction in nitrogen-status, and therefore less nitrogen available to support growth.

An intriguing result was obtained when 1 mM alanine was supplied with either 0.01 mM or 1 mM ammonium nitrate (Figure 3.10 C, D). Vegetative growth under these conditions was significantly inhibited in comparison with the growth of plants fed each nutrient alone. One would expect that if only nitrate uptake is inhibited in plants fed the combination of alanine and ammonium nitrate, then the vegetative growth of these plants should at least be equal to the growth of plants supplied only alanine. Since this is not seen, it could be indicative that another signalling pathway has been perturbed which results in the inhibition of alanine uptake at external concentrations of 1 mM. The predicted role of NRT1.1 in sensing of external nitrate is likely to interact with the nitrogen-status signalling pathway to regulate the supply and demand for nitrogen. Because of this interconnection, perturbing the nitrogen-status signalling pathway is likely to have a knock-on effect on NRT1.1 signalling. Perturbations in NRT1.1 signalling could have a direct or indirect effect on alanine uptake; or that altered NRT1.1 signalling could affect a yet undiscovered internal response which prevents the metabolism of alanine when concentrations reach 1 mM, and this altering the carbon to nitrogen ratio. Further investigations would be required to determine this.

Plants possess the ability to sense both carbon (Jang et al., 1997; Barker, 2000; Meyer et al., 2000; Schluepmann et al., 2003) and nitrogen metabolites (Remans et al., 2006; Castaings et al., 2009; Ho et al., 2009), which facilitates the regulation of metabolism and development in response to the ratio of carbon to nitrogen availability. For example, sugar-sensing enables plants to regulate photosynthesis in relation to the availability of carbon-skeletons (Jang et al., 1997), and nitrogen-status sensing enables plants to regulate nitrate uptake in response to levels of reduced or organic nitrogen (Ho et al., 2009). The two sensory pathways also appear to interact as, when carbon-skeletons are in abundance and internal nitrogen levels are low, transcripts associated with nitrogen assimilation increase in abundance (Cheng et al., 1992; Crawford, 1995). The opposite is also true that when

carbon-skeletons are less abundant, nitrogen assimilation is turned down. To this end, we know that feeding exogenous alanine to plants leads to an intracellular accumulation of alanine (Figure 3.6), which will undoubtedly affect the carbon to nitrogen ratio within the plant. There will likely be an excess of organic nitrogen (alanine), accompanied by a deficit in available carbon-skeletons because no extra carbon source is provided and photosynthesis will be limited to the amount of light available in the growth chamber. This perturbation in the carbon to nitrogen ratio could be the origin of the signal which results in a reduction of nitrate uptake and contributes to the growth phenotype observed.

The same inhibitory effect on vegetative growth is observed when glutamine or glutamate are supplied to roots together with ammonium nitrate (Figure 3.11) suggesting that the effect is not exclusive to alanine and may be common to many other amino acids. Nevertheless, alanine will be used in all future experiments as the inhibitory amino acid since the response to this amino acid is now well characterised in this growth-response assay.

3.3.3 Exogenous alanine reduces the abundance of transcripts associated with nitrate uptake and assimilation

Vegetative growth and nitrate uptake are inhibited by alanine, glutamine and glutamate (Figures 3.10 A – F and Figure 3.11). Owing to this it was reasonable to expect alterations at the transcript level to lead to the inhibition of nitrate uptake and growth. Indeed, when alanine is supplied, after 24 hours we see a significant repression of the nitrate transceptor NRT1.1, the high affinity nitrate transporter NRT2.2 and the NADH-dependent glutamate synthase enzyme. This further supports accumulating evidence that nitrate uptake and assimilation have been reduced at the transcript level, contributing to reduced nitrate uptake and ultimately, a reduction in growth.

Sensing and signalling of available external nitrate by the nitrate transceptor NRT1.1 triggers the induction of transcripts associated with nitrate transport and assimilation (Wang et al., 2003; Munos et al., 2004; Ho et al., 2009), which is collectively known as the primary nitrate

response (PNR) (Redinbaugh and Campbell, 1991). The NRT1.1 null mutant *chl1-5* is unable to initiate the PNR (Munos et al., 2004; Ho et al., 2009) which strongly suggests a role for this protein in the induction of the PNR. When we supply exogenous amino acids to plant roots, we see a strong reduction of the NRT1.1 transcript after 24 hours (Figure 3.11). A decrease in the NRT1.1 transcript likely leads to a decrease in the functional protein which could indicate that the sensory capacity of this protein has also been perturbed. If the capacity of NRT1.1 to initiate the PNR has been reduced, then nitrate uptake will also be markedly reduced, due to the reliance of some nitrate uptake transporters on induction by NRT1.1. The result would be nitrogen limited plants displaying an inhibition of growth, which is precisely what is observed when ammonium nitrate and alanine are supplied together (Figure 3.12).

The response of transcripts to alanine treatment was measured after 24 hours in these experiments. It is possible that repression of the relevant transcripts occurs very rapidly and is then sustained at a low level for the 24 hour period. It is therefore possible that an allosteric or other post-translational mechanism could be the primary response to the presence of alanine. To establish whether this is the case, transcripts would have to be measured over a time-course within the 24 hour period to identify how rapidly the repression occurs.

3.4 Summary

Alanine inhibits the vegetative growth of *Arabidopsis* supplied with ammonium nitrate. Whilst the mechanism controlling this response is currently unknown, it may be the result of perturbed nitrogen-status signalling. Exogenous alanine treatment results in a complex response that appears to alter nitrogen-status leading to repression of nitrate uptake and transcripts encoding nitrogen assimilation proteins, resulting in reduced growth. Exploiting this inhibitory response in a carefully designed reverse genetic growth-response screen of candidate knockout lines could enable the elucidation of components of the internal nitrogen

sensory pathway, which could then be characterised to establish whether they are indeed involved in nitrogen-status signalling.

Chapter 4: Identification of alanine-insensitive mutants in *Arabidopsis thaliana* using a reverse genetic approach.

4.1 Introduction

The aim of the work described in this chapter was firstly to isolate knockout mutants of candidate genes to screen, and secondly to conduct a growth screen under conditions that would reveal putative amino acid sensing mutants.

4.1.1 Reverse-genetics screening approach

Plant growth is variable which makes conducting a forward genetic screen for amino acid sensing mutants based on growth impossible (since relevant mutants are identified from the phenotype of individual plants in mutant populations). We did however find that taking the average growth of 12 plants led to reproducible growth measurements, meaning that a reverse genetic approach was more feasible. Reverse genetics, allows the phenotype caused by altering part of a known genetic sequence to be investigated. This is commonly achieved by T-DNA insertions which are incorporated randomly into the genome and disrupt the function of the allele at the insertion site. There are two widely available populations of T-DNA insertion mutants in *Arabidopsis*; SALK lines (Alonso et al., 2003), and SAIL lines (Sessions, 2002). The growth screen described in this chapter will make use of both of these mutant populations to obtain loss-of-function knockout mutants.

4.1.2 Selecting putative amino acid sensing mutants to screen

In order to conduct a reverse genetic screen, candidate genes must be selected. To maximise the likelihood of a successful screen, there must be a solid rationale for the involvement of the candidate genes in amino acid sensing. For that reason, in this screen, I focused on amino acid transporters as candidates for involvement in amino acid sensing. The reasons for this are twofold. Firstly, there is evidence that amino acids are sensed

internally and produce a 'nitrogen sufficiency' signal when they are in abundance. This nitrogen sufficiency signal induces a feedback loop that inhibits further nitrate uptake (Muller et al., 1995). In support of this is my findings that transcripts relating to nitrate uptake are inhibited in response to alanine gives a strong argument that amino acids levels are sensed by the plant. Since amino acid transporters specifically bind and transport amino acids, and are expressed throughout the plant, it is logical to expect that they may play a role in amino acid sensing.

The second line of evidence is that in mammals and yeast, amino acid transporter proteins have been identified as functioning as transceptors to sense amino acid status. These transceptors interact with signalling pathways to moderate cellular changes based on nitrogen-status. These changes include the activation of the TOR pathway to induce growth if amino acids are in abundance (Goberdhan et al., 2005; Rebsamen et al., 2015), or inducing transcription of amino acid and peptide transporters to promote amino acid uptake in response to the external availability of amino acids (Forsberg et al., 2008). A comprehensive review of amino acid transceptors is included in the introduction of this thesis, section 1.12.

For the reasons outlined above, the reverse-genetic growth screen in this chapter will focus on amino acid transporters as candidate genes to knock out.

4.2 Results

4.2.1 An outline of the screening process.

The screening process is outlined in Figure 4.1. Screening began once seed from homozygous T-DNA insertion lines had been collected. For each mutant line included in the screen, 24 plants were used. Half of the plants were fed 1 mM NH_4NO_3 as a control, and half were fed 1 mM alanine + 1 mM NH_4NO_3 for comparison with the control.

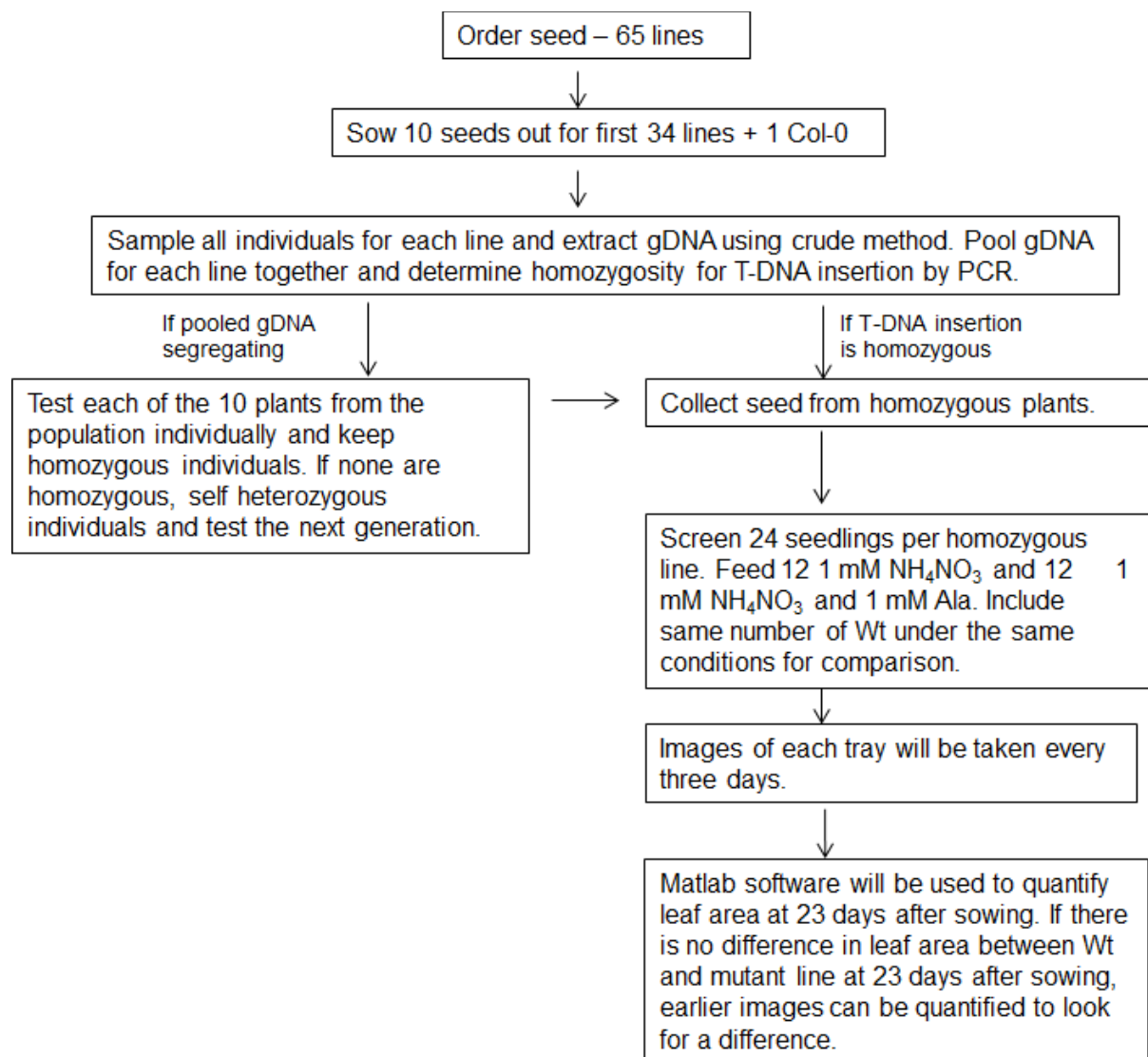


Figure 4.1. Flow diagram outlining the screening process.

4.2.2 Candidate gene selection process

Since there are > 60 amino acid transporters in Arabidopsis, it was necessary to prioritise the order in which to screen amino acid transporter loss-of-function mutants to maximise the chances of finding a nitrogen-status sensing mutant within the scope of the project. Based on two lines of evidence, I decided to prioritise amino acid transporters which are localised to the tonoplast. The first piece of evidence is the fact that the PATH transceptor in animals (Goberdhan et al., 2005), and the SLC38A9 transceptor in humans (Rebsamen et al., 2015) are both localised to the lysosome; the internal acidic environment of which is similar to the vacuole of plants. The second line of evidence comes from transgenic plants overexpressing alanine aminotransferase which are able to accumulate products of the reaction, such as alanine, whilst at the same time displaying an increased growth phenotype (Shrawat et al., 2008). In order to accumulate a product of a near equilibrium reaction (Duff et al., 2012), the product would have to be transported to another compartment, most likely the vacuole. This strongly suggests that the accumulated amino acid could be sensed at the vacuole, most likely via a tonoplast amino acid transporter. Since many amino acid transporters are poorly characterised in plants, determining with certainty those that are localised to the tonoplast will prove challenging. Consequently, in order to identify candidate tonoplast transporters, a combination of bioinformatic information and experimental evidence was used. The process of selecting candidate genes consisted of two main parts; a literature search to identify experimental evidence indicating where the protein is expressed and secondly, bioinformatic assessment of the presence of various sorting motifs in the protein amino acid sequence, particularly the N-terminal di-Leu or Tyr motifs in the protein sequence. These motifs have been shown to direct transporter proteins to the tonoplast or plasma membrane, respectively (Wolfenstetter et al., 2012). The localisation prediction software, WoLF PSORT (Horton et al., 2006), which makes predictions based on known sorting motifs was also used in addition to manual searches for di-Leu and Tyr motifs to ensure a greater accuracy. By using this strategy for prioritisation, all putative tonoplast

amino acid transporters should be identified and their respective knockouts used in the screen in a logical order.

Table 4.1 outlines the experimental and bioinformatic information available to support the subcellular localisation of amino acid transporters in Arabidopsis. All transporters that were likely to be localised to the tonoplast were prioritised over transporters localised elsewhere.

Table 4.1. Subcellular localisation of amino acid transporters in Arabidopsis

Accession Number	Transporter name	Evidence of tonoplast localisation	Evidence of PM localisation
AT1G58360	Amino acid permease 1 AAP1	No	Proteomics: (Benschop et al., 2007).
AT5G09220.1	Amino acid permease 2 AAP2	No	Predicted by SUBA (WoLF PSORT)
AT1G77380	Amino acid permease 3 AAP3	No	GFP: (Okumoto et al., 2004).
AT1G44100	Amino acid permease 5 AAP5	No	Predicted by SUBA (WoLF PSORT)
AT5G40780	Lysine/histidine transporter 1 LHT1	No	Proteomics: (Alexandersson et al., 2004).
AT1G48640	Lysine/histidine transporter-like 1 LHT-like 1	No	Proteomics: (Alexandersson et al., 2004; Mitra et al., 2009).
AT3G01760	Transmembrane amino acid transporter	No	Predicted by SUBA (WoLF PSORT)
AT1G25530	Transmembrane amino acid transporter-LHT9	No	Predicted by SUBA (WoLF PSORT)
AT4G35180	Lysine/histidine transporter 7 LHT-7	No	Predicted by SUBA (WoLF PSORT)
AT1G47670	Transmembrane amino acid transporter	No	Proteomics: (Benschop et al., 2007).
AT4G21120	Amino acid transporter 1 AAT1 (CAT1)	No	Proteomics: (Benschop et al., 2007).
AT1G58030	Cationic amino acid transporter 2 CAT2	Proteomics: (Jaquinod et al., 2007) GFP: (Su et al., 2004)	
AT5G36940	Cationic amino acid transporter 3 CAT3	No	Predicted by SUBA (WoLF PSORT)
AT3G03720	Cationic amino acid transporter 4 CAT4	Proteomics: (Carter et al., 2004; Jaquinod et al., 2007).	No
AT3G10600	Cationic amino acid transporter 7 CAT7	Predicted by SUBA (WoLF PSORT)	No
AT1G17120	Cationic amino acid transporter 8 CAT8	(Jaquinod et al., 2007).	No
AT1G05940	Cationic amino acid transporter 9 CAT9	(Jaquinod et al., 2007).	No
AT2G01170	Bidirectional amino acid transporter 1 BAT1	Predicted by SUBA (WoLF PSORT)	No
AT2G39130	AT2G39130	(Jaquinod et al., 2007).	Predicted by SUBA (WoLF PSORT)
AT2G40420	AT2G40420	(Jaquinod et al., 2007).	Predicted by SUBA (WoLF PSORT)

Accession Number	Transporter name	Evidence of tonoplast localisation	Evidence of PM localisation
AT2G41190	AT2G41190	(Jaquinod et al., 2007).	Predicted by SUBA (WoLF PSORT)
AT3G30390	AT3G30390	Proteomics: (Carter et al., 2004; Jaquinod et al., 2007)	Proteomic: (Mitra et al., 2009).
AT5G40670	AT5G40670	(Jaquinod et al., 2007).	No
AT5G65990	AT5G65990	No	Predicted by SUBA (WoLF PSORT)
AT2G42005	AT2G42005	No	Predicted by SUBA (WoLF PSORT)
AT5G02180	AT5G02180	No	Predicted by SUBA (WoLF PSORT)
AT5G15240	AT5G15240	Unclear	Unclear
AT2G02040	Peptide transporter 2	Proteomics: (Carter et al., 2004; Shimaoka et al., 2004; Dunkley et al., 2006; Jaquinod et al., 2007)	Predicted by SUBA (WoLF PSORT)
AT2G02020	Peptide transporter 4	GFP: (Weichert et al., 2012)	Predicted by SUBA (WoLF PSORT)
AT1G62200	Peptide transporter 6	GFP: (Weichert et al., 2012)	Predicted by SUBA (WoLF PSORT)
AT5G63850	Amino acid permease 4 AAP4	No	Predicted by SUBA (WoLF PSORT)
AT5G49630	Amino acid permease 6 AAP6	No	Proteomic: (Benschop et al., 2007).
AT5G23810	Amino acid permease 7 AAP7	No	Predicted by SUBA (WoLF PSORT)
AT1G10010	Amino acid permease 8 AAP8	No	Predicted by SUBA (WoLF PSORT)
AT1G24400	Lysine/histidine transporter 2 LHT2	No	Predicted by SUBA (WoLF PSORT)
AT1G67640	Lysine/histidine transporter-like 2 LHT-like 2	No	Predicted by SUBA (WoLF PSORT)
AT1G61270	Transmembrane amino acid transporter	No	Predicted by SUBA (WoLF PSORT)
AT1G71680	Transmembrane amino acid transporter	No	Predicted by SUBA (WoLF PSORT)
AT2G34960	Cationic amino acid transporter 5 CAT5	No	GFP: (Su et al., 2004).
AT5G04770	Cationic amino acid transporter 6 CAT6	No	Predicted by SUBA (WoLF PSORT)
AT2G39890	Proline transporter 1 ProT1	No	GFP: (Grallath et al., 2005).
AT3G55740	Proline transporter 2 ProT2	No	GFP: (Grallath et al., 2005).

Accession Number	Transporter name	Evidence of tonoplast localisation	Evidence of PM localisation
AT2G36590	Proline transporter 3 ProT3	No	GFP: (Grallath et al., 2005).
AT3G11900	Aromatic and neutral transporter 1 ANT1	No	Predicted by SUBA (WoLF PSORT)
AT5G55930	Oligopeptide transporter 1 OPT1	No	Predicted by SUBA (WoLF PSORT)
AT1G09930	Oligopeptide transporter 2 OPT2	No	Proteomics: (Benschop et al., 2007).
AT4G16370	Oligopeptide transporter 3 OPT3	No	Predicted by SUBA (WoLF PSORT)
AT5G64410	Oligopeptide transporter 4 OPT4	No	Proteomics: (Keinath et al., 2010).
AT4G26590	Oligopeptide transporter 5 OPT5	No	Predicted by SUBA (WoLF PSORT)
AT4G27730	Oligopeptide transporter 6 OPT6	No	Proteomics: Benschop, J. et al (2007).
AT4G10770	Oligopeptide transporter 7 OPT7	No	Proteomics: (Mitra et al., 2009).
AT5G53520	Oligopeptide transporter 8 OPT8	No	Predicted by SUBA (WoLF PSORT).
AT5G53510	Oligopeptide transporter 9 OPT9	No	Predicted by SUBA (WoLF PSORT).
AT1G48640	AT1G48640	No	Proteomics: (Alexandersson et al., 2004; Mitra et al., 2009).
AT3G09330	AT3G09330	No	Predicted by SUBA (WoLF PSORT)
AT2G41190	AT2G41190	(Jaquinod et al., 2007).	Predicted by SUBA (WoLF PSORT)
AT4G38250	AT4G38250 (path homologue)	No	Predicted by SUBA (WoLF PSORT)
AT3G28960	AT3G28960	(Jaquinod et al., 2007).	Predicted by SUBA (WoLF PSORT)
AT3G54140	Peptide transporter 1	No	GFP: (Dietrich et al., 2004). Proteomics: (Alexandersson et al., 2004; Mitra et al., 2009).
AT5G46050	Peptide transporter 3	No	Predicted by SUBA (WoLF PSORT)
AT5G01180	Peptide transporter 5	No	Predicted by SUBA (WoLF PSORT)

4.2.3 Genotyping SALK and SAIL knockout lines

The available Arabidopsis amino acid transporters knockouts were divided into two groups. The first group consisted of 34 lines that contained all the putative tonoplast localised transporter knockouts and the second group consisted of the remaining lines which would be screened if the first screen was unsuccessful. For each transporter, a minimum of two independent T-DNA insertion mutants were identified and one line was obtained for genotyping. It is essential to genotype all T-DNA insertion lines because heterozygous lines will contain a functioning allele which may alter the phenotype of the plant. In some cases, the functioning allele may be able to compensate for the loss-of-function of the allele containing the T-DNA insertion, which would conceal any effects of the loss of that allele. For this reason, all SALK and SAIL lines obtained were first genotyped and only homozygous lines were selected to be included in the screen. Figure 4.2 describes how the genotyping process was implemented.

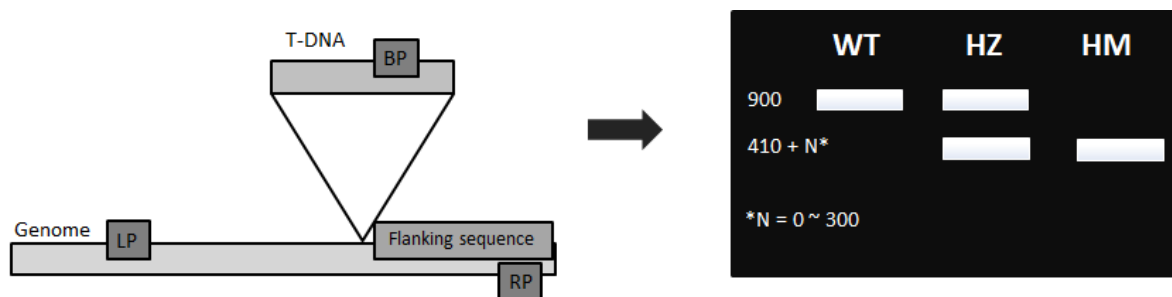


Figure 4.2. Schematic representation of the genotyping process.

Genotyping primers were selected from the SALK database. To detect the wild-type (WT) allele, primers specific to the gene of interests left border (LP) and right border (RP) were used. To detect the T-DNA insert, primers specific to the T-DNA backbone (BP) were used along with LP or RP depending on the orientation of the insert. WT = homozygotes for the WT allele, HM = homozygotes for the T-DNA allele and HZ = heterozygotes with both alleles.

A combination of three primers; BP + LP + RP, were used for genotyping SALK and SAIL lines (Figure 4.2). The LP + RP primer pair should produce a product of around 900-1100 bp for the WT allele and this product should be absent in the mutant allele. The BP + RP primer pair should produce a product of around 410 + N bp if the T-DNA insert is present. Mutants that are heterozygous for the T-DNA insert will produce both products (Figure 4.2).

Table 4.2 lists the outcome of the genotype analysis for the first 34 T-DNA insertion lines. Of the 34 lines included in the analysis, 30 were homozygous for the T-DNA insert and 4 were heterozygous. The 30 confirmed homozygous lines were used in the growth-response screen to try to capture components of the internal nitrogen sensing pathway.

Table 4.2 Genotype analysis of selected SALK and SAIL mutants by PCR.

Line no.	Gene	Line ID	Confirmed HM	Confirmed HZ
1	CAT2	SALK_097354C	Y	
2	CAT4	SALK_076699C	Y	
3	CAT7	SALK_141976C		Y
4	CAT8	SALK_052450C	Y	
5	CAT9	SALK_093929	Y	
6	BAT1	SALK_107641C	Y	
7	AT2G39130	SALK_109864C	Y	
8	AT2G40420	SALK_029814C	Y	
9	AT2G41190	SALK_043540C	Y	
10	AT3G30390	SALK_045966C	Y	
11	AT5G40670	SALK_068854C	Y	
12	AT5G65990	SALK_069516C	Y	
13	AT2G42005	SALK_025674C	Y	
14	AT4G38250	SALK_102614C	Y	
15	AT5G15240	SALK_009603C	Y	
16	ANT1	SALK_090394C		Y
17	PTR2	SALK_034312C	Y	
18	PTR4	SALK_062626C	Y	
19	PTR6	SALK_128040C	Y	
20	CAT1	SALK_143321C	Y	
21	CAT3	SALK_129691C	Y	
22	CAT6	SALK_082408C	Y	
23	AT5G02180	SALK_137967C	Y	
24	LHT1	SALK_083700C	Y	
25	LHT2	SALK_151610C		Y
26	LHT-like 1	SALK_114616C	Y	
27	AT3G01760	SALK_004201C	Y	
28	AT1G25530	SALK_009662C	Y	
29	AT4G35180	SALK_105583		Y
30	AT1G47670	SALK_201470	Y	
31	AAP1	SALK_078312C	Y	
32	AAP2	SALK_020688C	Y	
33	AAP3	SALK_148822C	Y	
34	AAP5	SAIL_193_D02	Y	
Total			30	4

4.2.4 Screening amino acid transporter knockout mutants for insensitivity to exogenous alanine

Homozygous T-DNA insertion lines were used in a growth-response screen to isolate mutants insensitive to alanine. For each line, 24 seedlings were grown on agar plates and divided into two groups of 12 plants. Each group were transplanted to a sand/vermiculite medium and watered every other day with an N-free nutrient solution. The first group were additionally supplied with 1 mM ammonium nitrate, and the second group were provided with 1 mM ammonium nitrate and 1 mM alanine (Figure 4.3).

The first round of screening revealed that a greater number of lines than expected were insensitive to the presence of alanine in the medium (Figure 4.3). The vegetative growth of these insensitive lines, 20 in total, showed no sign of growth inhibition when compared to wild-type, which showed inhibition of vegetative growth of approximately 42% in the presence of alanine. In one of the mutant lines, *at5g40670-1*, rosette growth in the presence of alanine was significantly greater than in the absence of alanine.

Due to the variable nature of plant growth, and the number of lines appearing to be insensitive to alanine, we decided to repeat the screen to test whether the same mutants would be insensitive to alanine a second time (Figure 4.4). The results indicate that 14 lines were insensitive alanine, 10 of which corroborate the first screen. All conditions were kept the same as previously, yet the vegetative growth of mutant lines was generally greater in the second screen compared with the first. For example, the greatest rosette area recorded in screen 1 was ~225 mm² whereas in screen 2 it was ~340 mm². The wild-type response to both feeding conditions, however, was comparable to the first screen (Figure 4.3). Despite this obvious variability between the two screens, the effect of alanine on rosette growth was consistent in ten mutant lines; *at5g02180*, *cat1-1*, *cat2-1*, *cat3-1*, *cat4-1*, *cat6-1*, *cat8-1*, *cat9-1*, *ptr2-1*, *ptr4-1*, *cat6-1*, *cat8-1* and *lht9-1*.

The screen was then repeated a third time (Figure 4.5), to be sure that the observed variability in the vegetative growth of the mutant lines between screens would not affect the growth-response to alanine. Again, mutant plant rosettes were generally larger than the wild-type under the same conditions, reminiscent of screen 2 (Figure 4.4). Nevertheless, 18 mutant lines were observed to be insensitive to alanine in screen 3, seven of which are consistent with the first and second screens. The seven lines were *lht9-1*, *at5g02180*, *cat1-1*, *cat2-1*, *cat6-1*, *cat8-1* and *ptr2-1*.

The concentration of ammonium nitrate used in all three screens was 1 mM. At concentrations of ammonium nitrate > 1 mM, the primary nitrate response (PNR) is induced by NRT1.1 to generate high affinity nitrate uptake in response to low external nitrate availability, and the opposite is true at concentrations < 1 mM (Wang et al., 2003; Munos et al., 2004; Ho et al., 2009). Thus, 1 mM ammonium nitrate is on the cusp between high and low nitrate concentrations that are perceived by plants, but it is generally acknowledged as a nitrogen-limiting concentration which induces high affinity nitrate uptake. Because this concentration may be on the tipping point between inducing high or low affinity uptake, we hypothesised that the variability in rosette size observed between screens could arise due to internal conditions driving the plant towards either high or low affinity nitrate uptake. In order to ensure that only low affinity nitrate uptake would be active, and therefore attempt to avoid potential variability due to affinity switching, we decided to repeat the screen a fourth time using a non-limiting 4 mM ammonium nitrate concentration. Before commencing the fourth screen, we had to first characterise the wild-type response to this higher ammonium nitrate concentration and 1 mM alanine (Figure 4.6), as this had not previously been done using this growth assay. Indeed, under a non-limiting 4 mM ammonium nitrate concentration, alanine as expected, significantly inhibits vegetative growth. This enabled us to repeat the screen a fourth time using non-limiting 4 mM ammonium nitrate (Figure 4.7). The result of this final screen suggests that the higher, non-limiting, ammonium nitrate concentration had no effect on decreasing the variability of rosette growth. We observed that 18 mutant lines were

insensitive to alanine, 4 of which corroborate with the first 3 screen results. These four lines were *lht9-1*, *cat2-1*, *cat6-1* and *cat8-1*.

Conversely, three lines were consistently sensitive to alanine in all four repetitions of the screen. The rosette growth of these three lines, which were *bat1-1*, *aap2-1* and *at2g391130*, was consistently inhibited by the presence of alanine in the media. Table 2 in Appendix C details the observed results in all four repetitions of the screen for comparison.

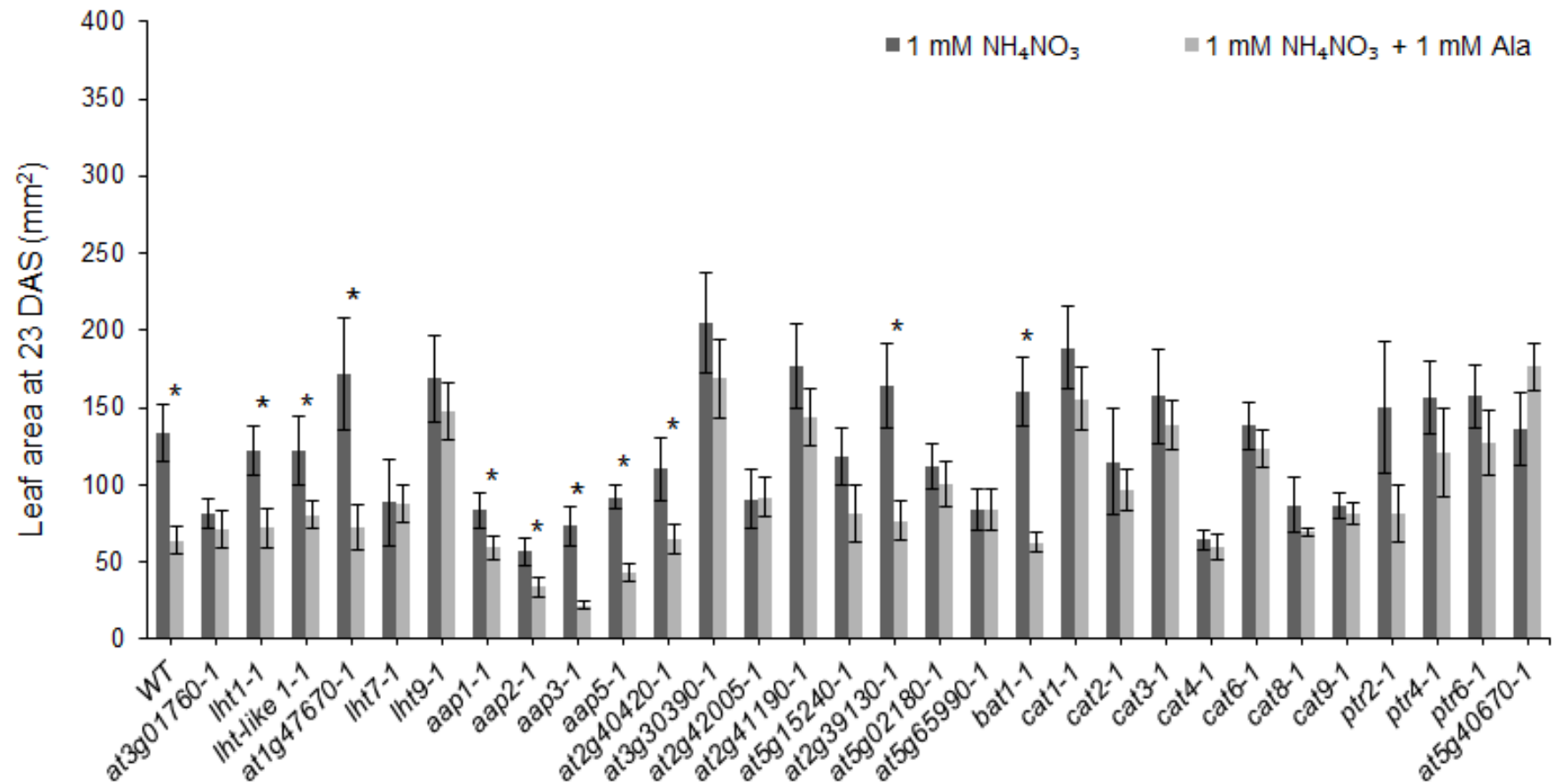


Figure 4.3. The effect of alanine on the vegetative growth of wild-type and amino acid transporter knockout lines.

Wild-type and mutant *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus either 1 mM NH₄NO₃ (dark bars) or 1 mM NH₄NO₃ + 1 mM alanine (light bars). Leaf area was quantified using Leaf lab software at day 23 after sowing. Data are the mean of $n = 12 \pm \text{SEM}$. Significant differences between + and minus alanine conditions within genotypes at $p < 0.05$ are indicated by * (t-tests).

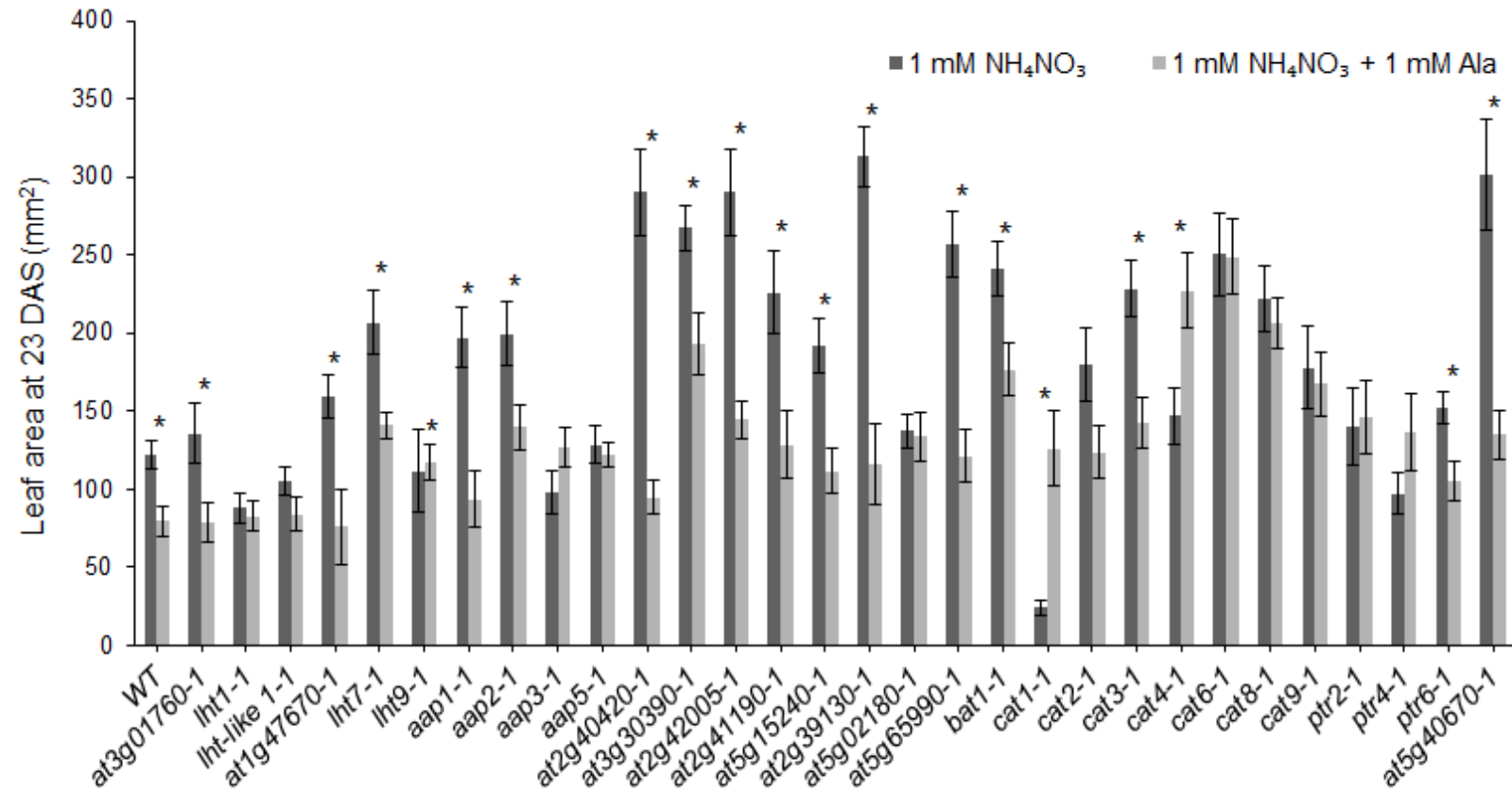


Figure 4.4. The effect of alanine on the vegetative growth of wild-type and amino acid transporter knockout lines: repeat screen 2

Wild-type and mutant *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus either 1 mM NH₄NO₃ (dark bars) or 1 mM NH₄NO₃ + 1 mM alanine (light bars). Leaf area was quantified using Leaf lab software at day 23 after sowing. Data are the mean of $n = 12 \pm \text{SEM}$. Significant differences between + and minus alanine conditions within genotypes at $p < 0.05$ are indicated by * (t-tests).

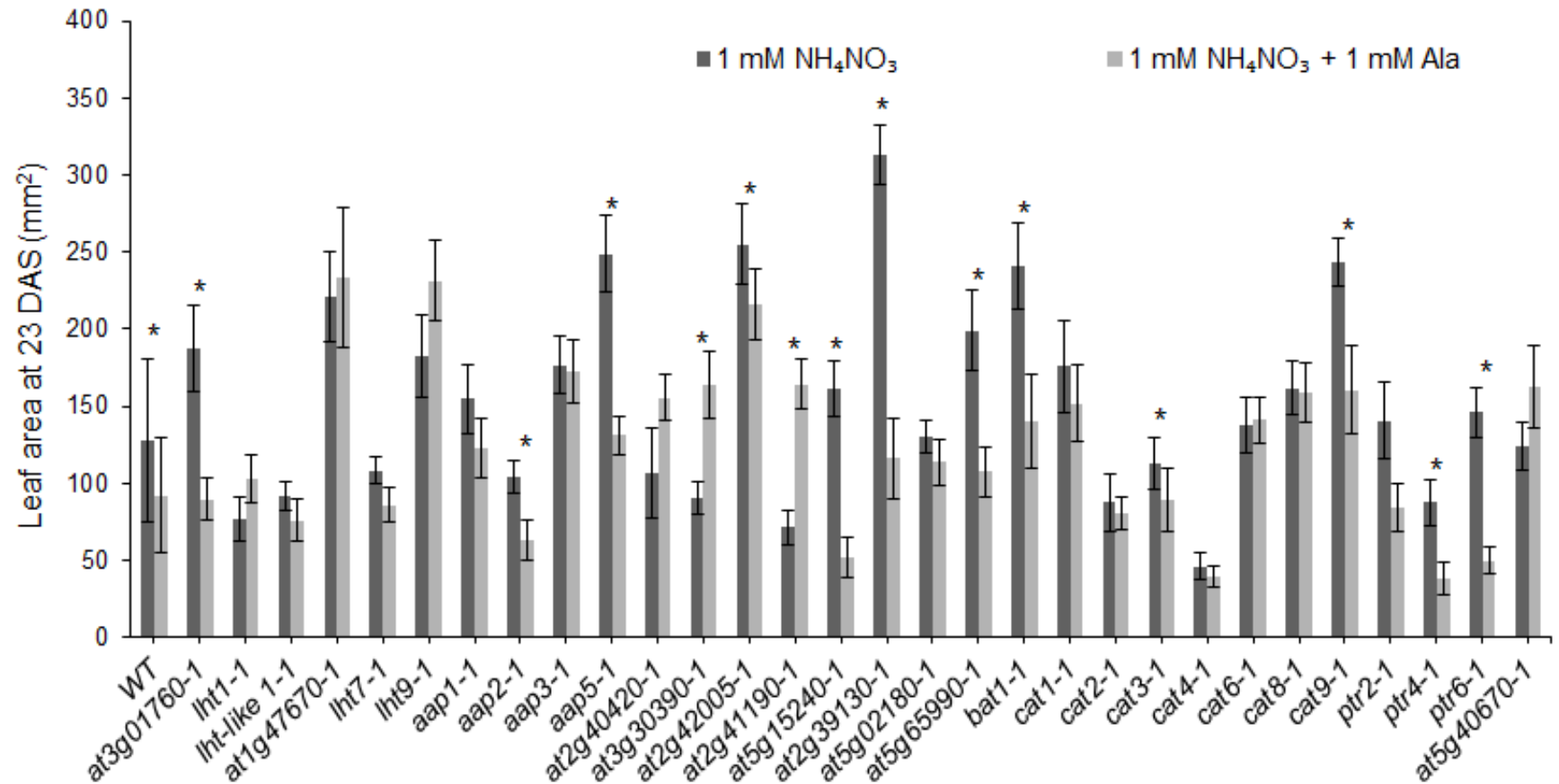


Figure 4.5. The effect of alanine on the vegetative growth of wild-type and amino acid transporter knockout lines: repeat screen 3

Wild-type and mutant *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus either 1 mM NH₄NO₃ (dark bars) or 1 mM NH₄NO₃ + 1 mM alanine (light bars). Leaf area was quantified using Leaf lab software at day 23 after sowing. Data are the mean of $n = 12 \pm \text{SEM}$. Significant differences between + and minus alanine conditions within genotypes at $p < 0.05$ are indicated by * (t-tests).

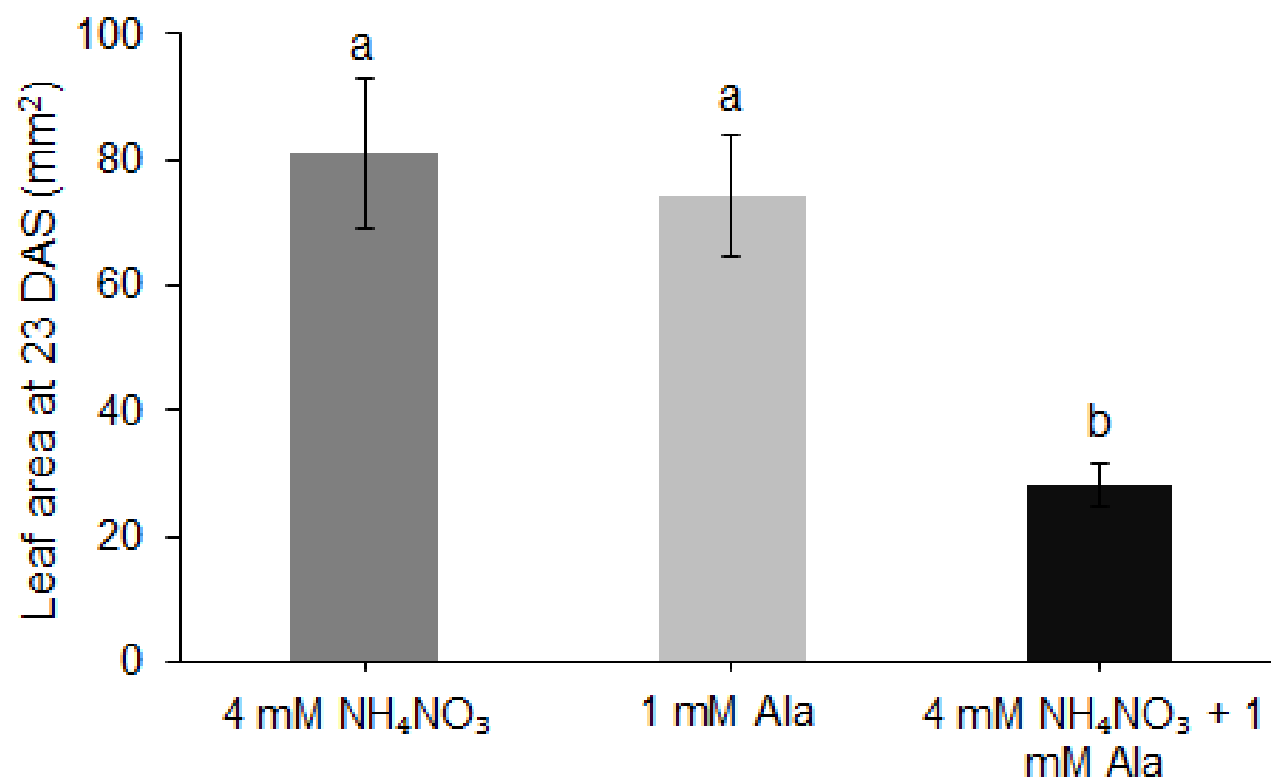


Figure 4.6. The wild-type growth response to exogenously supplied, non-limiting, 4 mM NH₄NO₃ + 1 mM alanine.

Wild-type *Arabidopsis* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus the indicated concentrations of alanine and/or NH₄NO₃. Leaf area was quantified using Leaf lab software at day 23 after sowing. Data are the mean of $n = 12 \pm \text{SEM}$. Values significantly different from each other are indicated by letters ($p < 0.05$, t-tests).

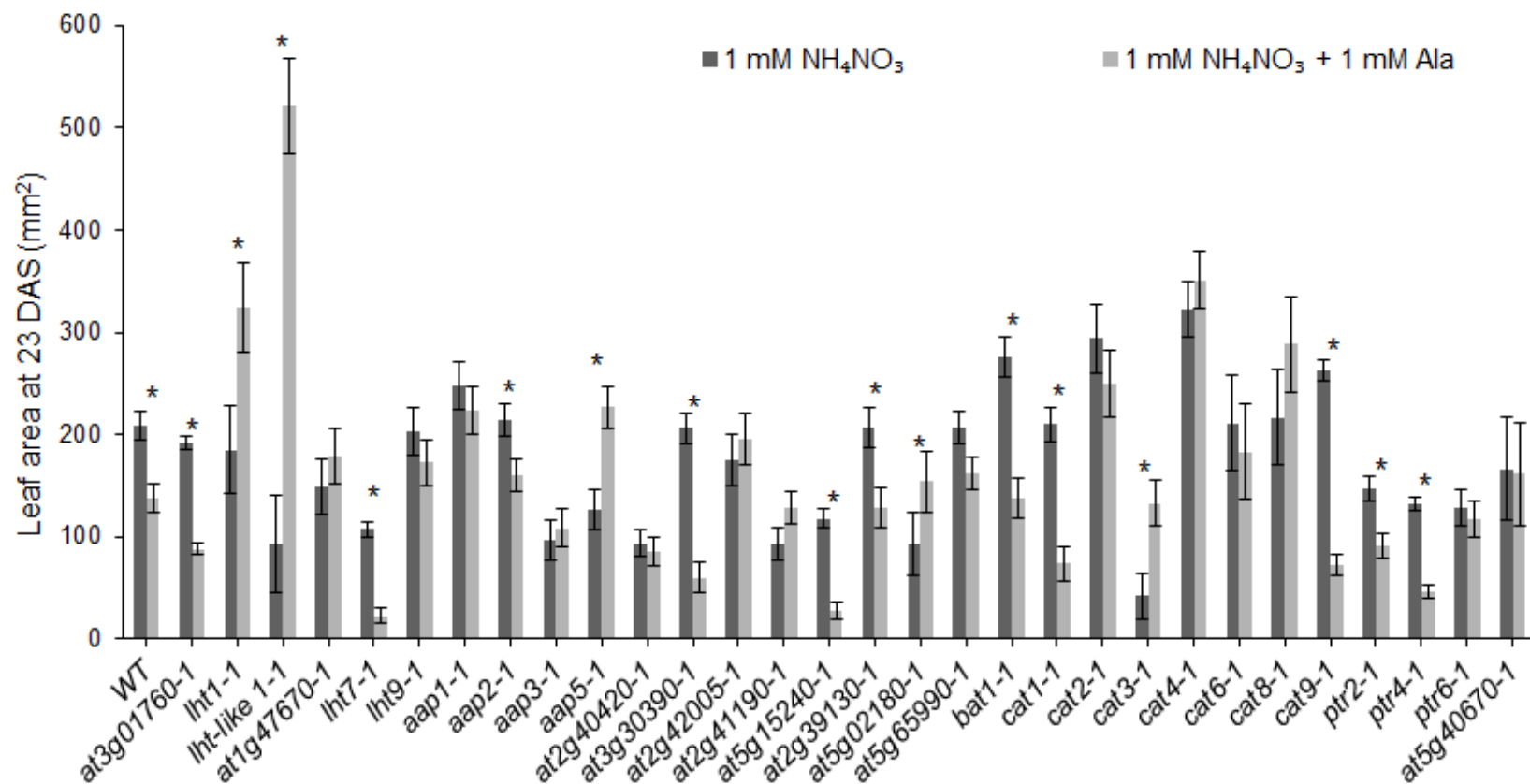


Figure 4.7. The effect of alanine on the vegetative growth of wild-type and amino acid transporter knockout lines: repeat screen 4 using 4 mM NH₄NO₃.

Wild-type and mutant Arabidopsis seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus either 4 mM NH₄NO₃ (dark bars) or 4 mM NH₄NO₃ + 1 mM alanine (light bars). Leaf area was quantified using Leaf lab software at day 23 after sowing. Data are the mean of n = 12 ± SEM. Significant differences between + and minus alanine conditions within genotypes at p<0.05 are indicated by * (t-tests).

4.3 Discussion

4.3.1 The response of the mutant rosette growth was variable between screens.

The vegetative growth of plants compared between screens was variable. For example, the vegetative growth of *at3g30390-1* in the presence of alanine was significantly greater than wild-type in screen 3 (Figure 4.5), but not significantly different in screens 1 and 2 (Figures 4.3 and 4.4), and significantly reduced in screen 4 (Figure 4.7). This variability likely arises because plant growth is a complex process which is controlled by many factors. Examples of environmental factors which affect growth include light (Morelli and Ruberti, 2002), temperature (Balasubramanian et al., 2006), humidity (Dow et al., 2014), radiant energy (Soler et al., 2003), soil aeration and structure (Unger and Kaspar, 1994), as well as biotic factors. Whilst every attempt was made to keep all these factors consistent across all screens by using a controlled environment chamber, and sterilised growth media, it is likely that some differences occurred. For instance, it is likely that the level of aeration in the sand and vermiculite mixture differed between trays and between batches as this was hard to keep constant. This could have affected the structure of the sand and its ability to retain the nutrient medium, both of which would have an impact on growth. It is also impossible to state with confidence that biotic factors did not affect growth. The sand and vermiculite used were sterile and plants were germinated on sterile agar plates, and then placed in to a clean growth cabinet. However, the cabinet and the surrounding environment were not sterile. I observed that the nutrient medium, which was placed in the bottom of the tray containing the plants, would regularly become contaminated with unknown contaminants in the warm, humid environment of the chamber. This could have caused abiotic stress on the plants and consequently affected growth and added to the variability observed.

4.3.2 The rosette growth of wild-type remained constant across all screens

A remarkable observation was that unlike most of the mutants, the vegetative growth of wild-type plants did not vary between screens. The inhibitory response of alanine on wild-type rosette growth was consistent throughout and the rosette size did not vary considerably between screens. This suggests that the varying responses of most of the mutants to alanine were unique to the mutants and might be related to the existence of an integrated signalling network. The loss of an amino acid transporter may alter the local pool of amino acids, which could potentially perturb an integrated signalling network that controls nitrogen-status signalling. The change in local amino acid availability is likely to be sensed and communicated to the nitrogen-status signalling network. These changes are likely to have an impact on growth, but this may be variable depending on the overall status of the plant as well as all other factors influencing growth. Mutant rosette growth variability could also arise because the mutant lines are more sensitive to the concentration of ammonium nitrate being fed. 1 mM ammonium nitrate is on the cusp of inducing the switch from high to low affinity nitrate uptake. The amino acid transporter knockouts may be affected in the ability to respond appropriately to this concentration, and other internal factors may contribute to whether high or low affinity uptake is induced and hence contributes to the variability seen. In order to test these ideas, it would be necessary to compare the growth variability of other T-DNA insertion mutants that do not affect amino acid transport or metabolism.

4.3.3 Four amino acid transporter knockouts are consistently insensitive to exogenous alanine

Four mutant lines were consistently insensitive to alanine in all four repetitions of the growth-response screen. These mutants contain a T-DNA insertion in the amino acid transporters CAT2 (*cat2-1*), CAT6 (*cat6-1*), CAT8 (*cat8-1*) and LHT9 (*lht9-1*). Because of the consistent insensitivity to alanine, we hypothesise that these four mutants are either defective for alanine uptake or are indeed perturbed in nitrogen-status sensing. Since there are numerous

amino acid transporters that could transport alanine, it seems unlikely that removing just one transporter would result in an inability to take up this amino acid. Therefore, it is more likely that these lines might be nitrogen-status sensory mutants. Signalling for increased availability of alanine may have been perturbed in these mutants to such an extent that the normal feedback inhibition of nitrate uptake and assimilation in response to amino acid accumulation has been severely disrupted. Thus resulting in uninhibited nitrate uptake as well as alanine uptake, this would contribute to increased nitrogen-status and therefore increased availability of nitrogen. This would potentially explain the growth phenotype observed. As a result, all four mutant lines will be taken forward and put through a round of secondary screening to test their putative involvement in nitrogen-status sensing.

4.4 Summary

Four replications of a reverse genetic growth-response screen of *Arabidopsis* to exogenous alanine revealed that four out of 30 amino acid transporter knockout mutants were consistently insensitive to the presence of exogenous alanine. These mutants may be perturbed in nitrogen-status sensing. Molecular analysis will be presented in the subsequent chapter to address this.

Chapter 5: Secondary screening and further characterisation of the alanine-insensitive mutants

5.1 Introduction

The aim of the work described in this chapter was primarily to determine whether mutants isolated in the reverse-genetic screen described in chapter 4 were insensitive to alanine because they are unable to take it up, or because they are potentially perturbed in nitrogen-status signalling. A series of secondary screens were designed to investigate this. The secondary aim of this chapter was to test the potential origin of the signalling perturbation in the mutants. I hypothesised that a common metabolic signature may be produced in the alanine insensitive mutants, which interferes with the feedback repression of nitrate uptake, resulting in the observed growth insensitivity to exogenous alanine. Extensive metabolite profiles were analysed to investigate whether common metabolite perturbations were present in the four mutant lines.

5.1.1 Secondary screening

There are two potential mechanistic explanations to account for the alanine-insensitive growth phenotype observed during the growth-screen in chapter 4. Perhaps the most obvious is that, owing to these mutants being amino acid transporter knockout mutants, it is feasible that the capacity for the uptake of exogenous alanine has been greatly reduced. Since there are greater than 60 amino acid transporters in Arabidopsis it seems unlikely that the loss-of-function of one transporter would affect the uptake rates of alanine. However, in a similar study to isolate mutants that were insensitive to exogenous arginine, an amino acid transporter knock-out mutant was isolated (*aap5*) (Svennerstam et al., 2008). Further investigation into amino acid uptake rates revealed that the plants had a greatly reduced capacity for arginine uptake, thus explaining the insensitivity phenotype and identifying a role for AAP5 in arginine acquisition. Similarly, the LHT1 knockout (*lht1*) mutant was unable to

survive on media containing glutamate or aspartate as the sole source of nitrogen. Therefore, indicating a major role for LHT1 in root glutamate and aspartate acquisition (Hirner et al., 2006). This clearly demonstrates that the loss-of-function of an amino acid transporter can affect amino acid uptake rates, and therefore this requires investigation to ensure the correct interpretation of the alanine-insensitive mutant growth phenotype. Consequently, one of the experimental aims of this chapter was to conduct ^3H -alanine uptake feeding experiments in wild-type plants and the alanine-insensitive mutants to rule out significant reductions in the capacity for alanine uptake in the mutants.

Alternatively, if alanine uptake rates are normal in the mutants, the alanine-insensitive phenotype can be explained due to perturbed nitrogen-status signalling. To clarify this I measured the abundance of transcripts related to nitrate uptake. Ordinarily, in wild-type *Arabidopsis*, the *NRT1.1* transcript abundance is decreased in response to exogenous alanine. This is most likely because increased alanine levels are sensed as nitrogen sufficiency, activating a signalling pathway which transduces the nitrogen sufficiency signal to ultimately reduce further nitrate uptake. If the alanine-insensitive mutants are perturbed in nitrogen-status signalling, one might expect that *NRT1.1* transcripts are unchanged in response to exogenous alanine.

5.1.2 Metabolic signature hypothesis

An unanticipated number of amino acid transporter knockout mutants were observed to have growth phenotypes that were insensitive to exogenous alanine. This was unexpected for a number of reasons. Firstly, the screen was initiated in the anticipation of identifying transceptors and in other examples of transceptors, for example *NRT1.1* in *Arabidopsis* (Ho et al., 2009), *PATH* in *Drosophila* (Goberdhan et al., 2005) and *SLC38A9* in humans (Rebsamen et al., 2015), it has been found that one gene encodes for the transceptor, and that the loss-of-function of the gene results in a phenotype that can be associated with the loss of signalling function. Thus implying that no other gene can compensate for the loss-of-

function. It was therefore quite unexpected that the growth-screen isolated four alanine-insensitive mutants, rather than one mutant which could have indicated a transceptor-like function. Consequently, I have formulated two hypotheses to explain how the four alanine-insensitive mutants could produce the same signalling phenotype.

The first hypothesis posits that there is a perturbation in metabolite levels, caused by the loss-of-function of the transporter and that some of these changes are common to all four mutants. It is conceivable that this common metabolite change acts as a metabolic signal, which overrides normal nitrogen-status signalling to prevent the suppression of the *NRT1.1* transcript (Figure 5.1). Knocking out an amino acid transporter is likely to alter the subcellular distribution of amino acids as well as other metabolites. This subcellular perturbation in metabolite levels may act as a signal for insufficient nitrogen uptake in relation to demand, which overrides the normal nitrogen sufficiency signal produced by high levels of amino acids. Why this is unique to the four alanine insensitive mutants and not to the other amino acid transporters screened is unclear. It could perhaps be related to the localisation of these particular transporters, or to the substrates they transport.

To test the metabolic signature hypothesis, this chapter will include metabolite profiling of the alanine-insensitive mutant lines as well as wild-type. Both GC-MS and LC-MS metabolite profiling was done to maximise the number of metabolites analysed and thereby provide the broadest possible test of the metabolite signature hypothesis. Comparisons between mutant and wild-type will be made to ascertain whether there are differences common to the mutants but absent in the wild-type. This will enable the identification of putative metabolic signatures which could be the origin of the signal which perturbs nitrogen-status signalling.

The second hypothesis is that the four transporter proteins could form a functional complex, thus the removal of each protein in turn results in the same growth phenotype. This hypothesis will be addressed further in chapter 6.

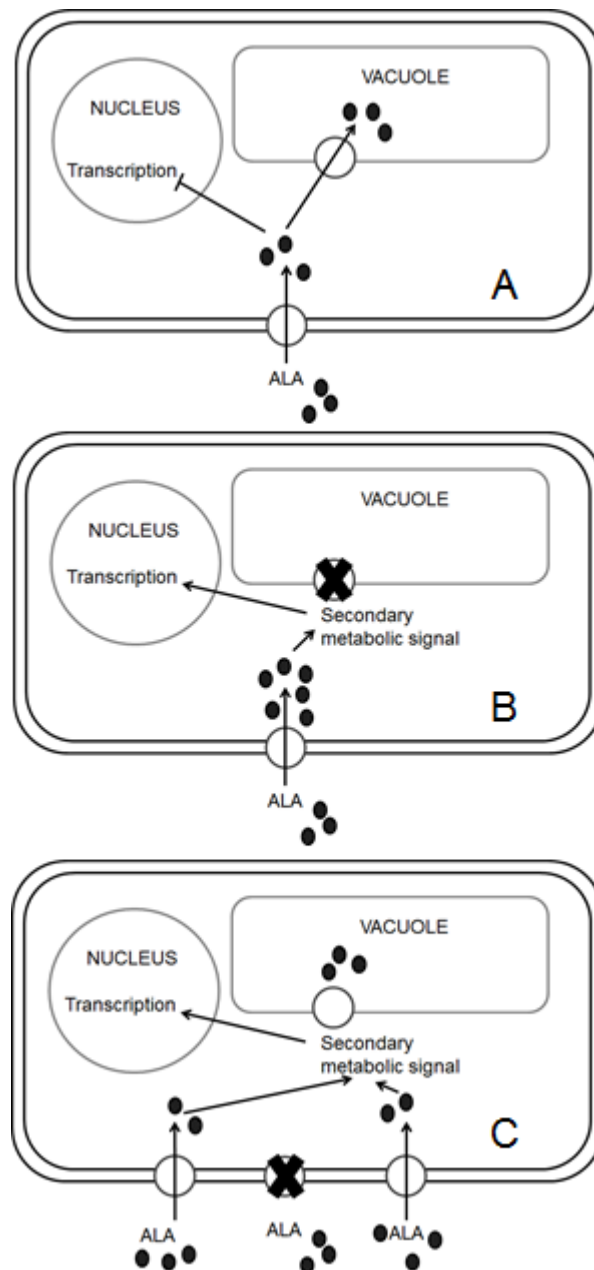


Figure 5.1. Schematic diagrams explaining how a secondary metabolic signal might be generated to alter transcription in the alanine insensitive mutants.

Schematic A depicts the normal plant cell: Exogenous 1 mM alanine is transported into the cell, where it accumulates and a nitrogen-sufficiency signal is produced which suppresses *NRT1.1* transcription. In schematic B, the loss of a tonoplast transporter, i.e. CAT2 and CAT8, has altered localised metabolite levels, which has generated a signal to override the inhibition of the *NRT1.1* transcript. In schematic C, the loss of a plasma membrane transporter, i.e. CAT8 and CAT6 has had the same effect on localised metabolite levels leading to the same derepression of *NRT1.1*.

5.2 Results

5.2.1 Using RT-PCR to detect transcripts in alanine insensitive mutants

T-DNA insertions are short DNA sequences which incorporate randomly into the plant genome and often completely disrupt transcription to result in a knockout mutant (Alonso, 2003). However, there are some incidences in which partial or full transcripts can still be produced. For example, if the insert is toward the 3' end, sometimes a truncated transcript can be produced (Wang, 2008). Equally, full length transcripts can result if the T-DNA insertion point is before the start codon or after the stop codon. Both of these examples fail to produce absolute loss-of-function mutants, which is why it is essential to confirm the status of the transcript in T-DNA knockout lines so that we can be sure that any phenotype produced can be associated with the loss-of-function of that gene.

Standard RT-PCR was used to detect the presence of full-length transcripts in the alanine-insensitive T-DNA mutants. Primers for Arabidopsis TIP41-like transcript were used as a reference transcript, as has previously been shown to be present at stable levels in Arabidopsis across a variety of conditions (Czechowski et al., 2005).

No detectable transcripts were observed in lines *cat2-1* and *lht9-1* (Figure 5.2 B and E). A faint band was visible when CAT6 primers were used with *cat6-1* cDNA indicating that this line is most likely a knock down (Figure 5.2 D). The T-DNA insertion site in *cat6-1* is at the 3' end (Figure 5.2 A), which suggests that transcription could still occur, however, the RT-PCR result indicates that it occurs at lower than normal rates. A band was also produced with CAT8 primers and *cat8-1* cDNA and this band appears much brighter than that produced by the loading control (Figure 5.2 C). From this we can surmise that *cat8-1* has a detectable transcript, and that this transcript may be overexpressed. The T-DNA insertion site in *cat8-1* is in the 5' UTR (Figure 5.2 A), a region which commonly houses the gene promoter; therefore it is possible that the T-DNA sequence, which is driven by a 35-S promoter, has

disrupted the CAT8 gene promoter and caused the transcript to overexpress. It would however, be necessary to use quantitative PCR to determine this with better accuracy.

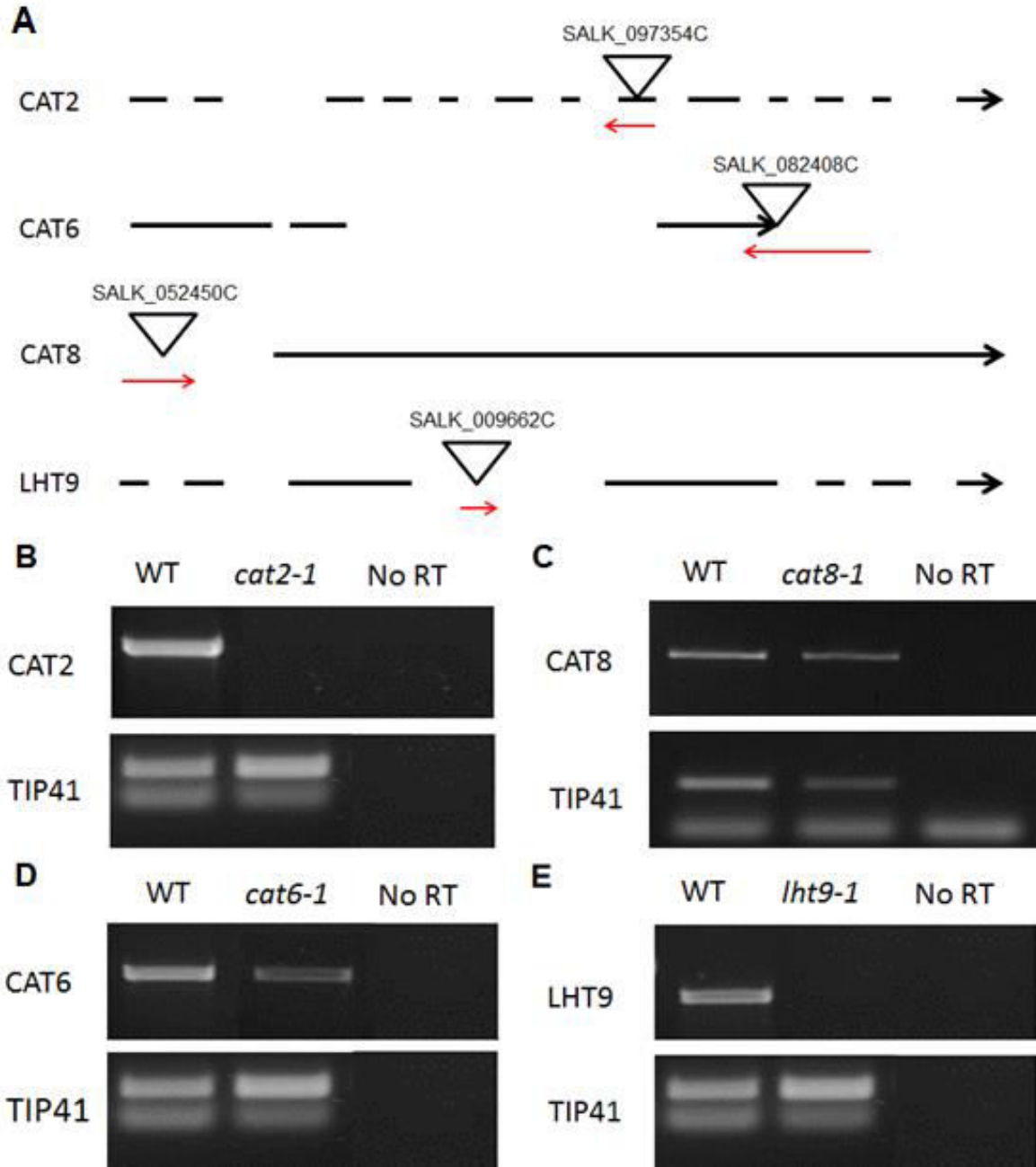


Figure 5.2. RT-PCR to detect transcripts in alanine insensitive SALK mutants.

Plants were grown on $\frac{1}{2}$ MS plates and harvested at 11 days old for cDNA synthesis. Primers were designed to amplify full length gene transcripts (Table 2.2) and Arabidopsis TIP41-like transcript was included as a loading control. No RT = no reverse transcriptase to control for genomic DNA contamination. Schematic representation of the location of the respective T-DNA inserts (A) (black lines = exon, red lines = T-DNA insert); WT and *cat2-1* cDNA with CAT2 and TIP41 primers (B); WT and *cat8-1* cDNA with CAT8 and TIP41-like primers (C); WT and *cat6-1* cDNA with CAT6 and TIP41 primers (D); and WT and *lht9-1* cDNA with LHT9 and TIP41 primers (E).

5.2.2 Measuring alanine uptake rates in alanine insensitive mutants

Amino acid transcript abundance is altered in the alanine-insensitive mutants, raising the possibility that the insensitivity to exogenous alanine is caused by an inability to take up alanine at normal rates. I therefore designed a labelled alanine feeding experiment in order to rule out this prospect.

^3H -alanine was fed to wild-type and mutant lines and the uptake rate of ^3H -alanine over a 5 minute time period was measured (Figure 5.3). The 5 minute time period was selected after conducting a preliminary timecourse experiment. This revealed that alanine was steadily taken up between 0 and 15 minutes, but that uptake plateaued after 15 minutes. In order to avoid the plateau in uptake, and therefore gain a more accurate rate of alanine uptake, 5 minutes was the time period selected for analysis.

^3H -alanine feeding experiments suggest that alanine-insensitive mutants take up alanine at the same rate as wild-type plants (Figure 5.3). The addition of 1 mM NH_4NO_3 to the medium also had no significant effect on the rate of ^3H -alanine uptake (Figure 5.3).

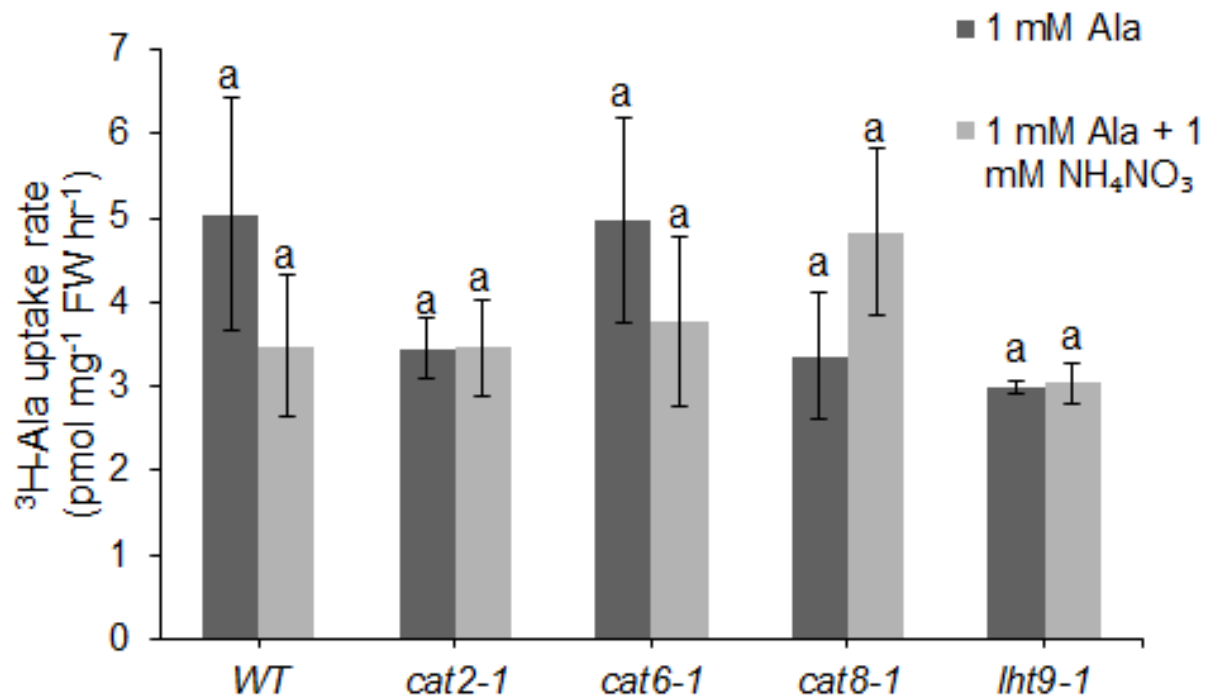


Figure 5.3. ³H-Ala uptake in alanine insensitive mutants and wild-type.

Plants were grown on ½ MS plates. After 11 days, 10 seedlings per genotype were placed in to a flask containing 0.1 mM CaCl₂. After 24 hours, plants were removed, blotted dry and resuspended in 5 ml of uptake medium (either 1 mM alanine or 1 mM NH₄NO₃ + 1 mM alanine). 0.038 MBq of ³H-Alanine was added and flasks were agitated at room temperature. Whole seedlings were sampled at 0 and 5 minute time points. Data are the mean of n = 3 ± SEM. Letters indicate significant differences of mutant lines relative to wild-type at P<0.05 (t-tests).

5.2.3 Measuring the abundance of nitrate transporter transcripts in response to exogenously supplied alanine in alanine-insensitive mutant lines.

The rate of alanine uptake in the alanine-insensitive mutants is unaffected, suggesting that it is not the inability to take up alanine that causes insensitivity to exogenous alanine. The alternative cause for alanine insensitivity may be due to perturbed nitrogen-status sensing. To investigate this, I measured the abundance of the nitrate transporter transcripts *NRT1.1*, *NRT2.1* and *NRT2.2* (Figure 5.3). *NRT1.1* and *NRT2.2* transcripts were previously observed to decrease in response to exogenous alanine supplied to wild-type plants (Chapter 3, Figure 3.10). The abundance of the *NRT2.1* transcript was not significantly altered in response to exogenous alanine in wild-type. However, this transcript was measured in the

mutants as it is an important high-affinity nitrate transporter which is induced by NRT1.1 as part of the primary nitrate response (PNR).

The abundance of the *NRT1.1* transcript in mutants supplied with alanine was greatly increased in all four lines when expressed relative to the untreated (- alanine) control (Figure 5.4 A). This was in contrast to wild-type, in which a decrease was observed (Figure 5.4 A). *NRT2.1* transcript abundance was also increased compared to wild-type in two of the mutant lines; *lht9-1* and *cat2-1*, and not significantly different from the untreated control in *cat6-1* and *cat8-1* (Figure 5.4 B). Lastly, when *NRT2.2* transcripts were measured, three mutant lines; *cat2-1*, *cat6-1* and *cat8-1* showed an induction of this transcript compared to the wild-type abundance, whereas no significant differences in transcript abundance was observed for *lht9-1* relative to alanine treatment or wild-type levels (Figure 5.4 C). Collectively these data show that normal feedback repression of nitrate transporter transcripts has been disrupted, thus indicating that the alanine-insensitive mutants may be perturbed in normal nitrogen-status sensing.

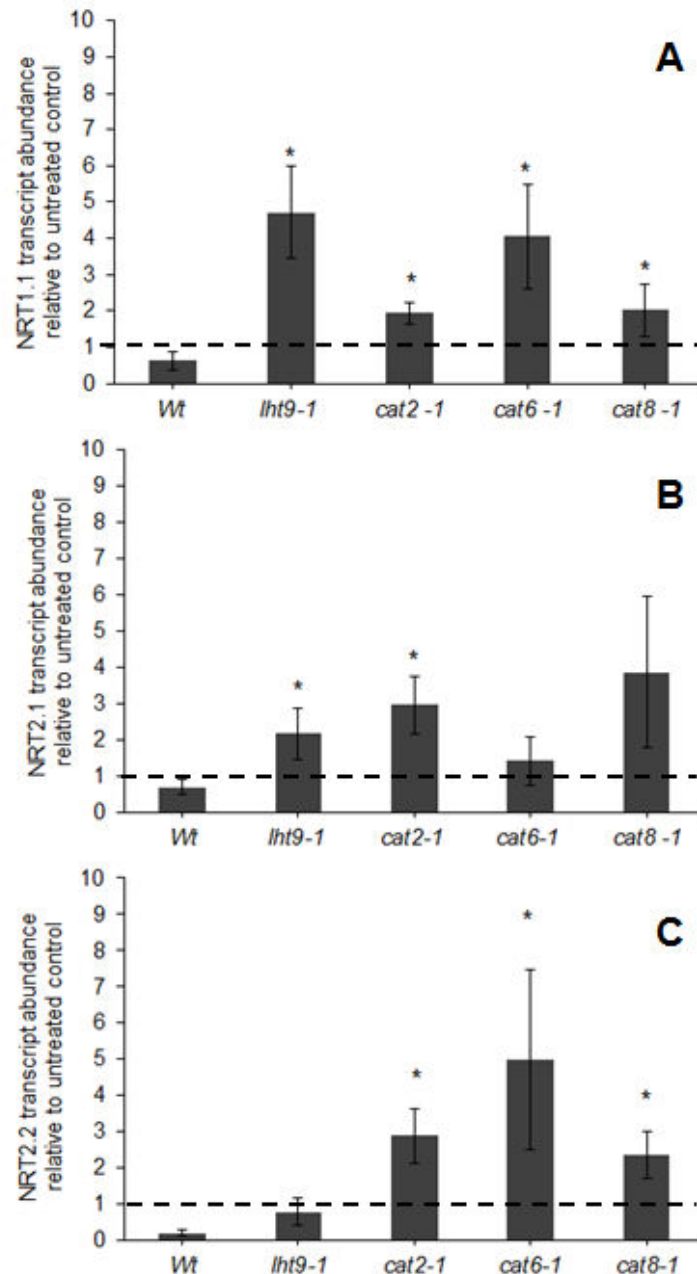


Figure 5.4. The effect of exogenously supplied alanine on *NRT* transcript abundances in wild-type and alanine insensitive mutants.

Wild-type and mutant *Arabidopsis* seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were starved for 24 hours in flasks containing 0.1 mM CaCl_2 . After 24 hours, 1 mM NH_4NO_3 (untreated control) or 1 mM NH_4NO_3 + 1 mM Ala was added for a further 24 hours. Roots were sampled exactly 24 hours later for RNA extraction. RNA was transcribed to cDNA for qRT-PCR analysis. Transcript abundance was normalised to the housekeeping transcript *TIP41-like* and expressed relative to the untreated control (1 mM NH_4NO_3). Abundance of NRT1.1 transcript in wild-type and mutants (A); Abundance of NRT2.1 transcript in wild-type and mutants (B); Abundance of NRT2.2 transcript in wild-type and mutants (C). Data are the mean of $n = 3 \pm \text{SEM}$. Black dashed line represents the untreated control value. Values significantly different from wild-type at $p < 0.05$ are indicated by * (t-tests).

5.2.4 Metabolite profiling

In order to investigate the potential origin of the signal that causes the perturbation in nitrogen-status signalling in alanine-insensitive mutants, I measured metabolite levels in the roots of mutants fed with exogenous alanine and compared them to wild-type and *bat1-1* levels (Figure 5.5). The alanine-sensitive mutant, *bat1-1* was included as a negative control. GC-MS was used to quantify metabolites from methanol extracted root samples and Tagfinder was used to analyse the data (Luedemann et al., 2008). A full table of the results can be found in Appendix D Table 3.

To identify individual metabolite differences between genotypes I analysed the data using t-tests between each line and wild-type. To control for multiple test errors, I set the false positive discovery rate (q-value) to 5%. This is important because in a large data set such as a metabolite profile, where 1000 measurements might be made, using the standard p value cut-off of $p < 0.05$ could result in 50 metabolites being deemed significant by chance. In other words, larger datasets proportionally increase the rate of false positives. Setting a false positive discovery rate controls for the expected proportion of false positives among the significant results (Storey, 2003). Table 5.1 lists the significantly different metabolites identified using this method.

Table 5.1. Metabolites significantly different from wild-type.

Values were calculated as significantly different using t-tests ($p < 0.05$) and correcting for false discovery rates using a q-value of 3%

Genotype	Metabolites sig. increased compared to WT	Metabolites sig. decreased compared to WT
<i>bat1-1</i>	0	0
<i>cat2-1</i>	Alanine 3-cyano- (2TMS) Mevalonic acid-1.5-lactone (1TMS) Ornithine-1.5 lactam (2TMS)	0
<i>cat6-1</i>	Ascorbic acid (4TMS) Ornithine-1.5 lactam (2TMS)	0
<i>cat8-1</i>	Mevalonic acid-1.5-lactone (1TMS) Ornithine-1.5 lactam (2TMS) Threose (1MEOX) (3TMS) MP	Asparagine (3TMS) Cysteine. N-acetyl- (3TMS) Pyruvic acid (2TMS)
<i>lht9-1</i>	Mevalonic acid-1.5-lactone (1TMS) Ornithine-1.5 lactam (2TMS) Threose (1MEOX) (3TMS) MP	Asparagine (3TMS) Cysteine. N-acetyl- (3TMS) Pyruvic acid (2TMS) Succinic acid (2TMS)

TMS = trimethylsilyl derivatives, MEOX = O-methylhydroxylamine hydrochloride derivatives.

Interestingly, *bat1-1*, the alanine-sensitive mutant included as a negative control has no metabolites significantly different from wild-type (Table 5.1). One metabolite, ornithine-1.5 lactam was consistently more abundant in all the alanine-insensitive mutants compared to wild-type. Mevalonic acid-1.5 lactone was increased in *cat2-1*, *cat8-1*, and *lht9-1*. Almost identical metabolite changes were observed in *cat8-1* and *lht9-1*, with the only additional difference being significantly reduced succinic acid in *lht9-1*. Ascorbic acid was significantly increased in *cat6-1*, whereas 3-cyano-alanine was increased in *cat2-1* compared to wild-type.

There were some important metabolites, mostly glycolytic intermediates that GC-MS failed to detect. I therefore used LC-MS and a spectrophotometric assay to detect a wider range of metabolites in the mutants and wild-type (Figure 5.5 and Table 5.2).

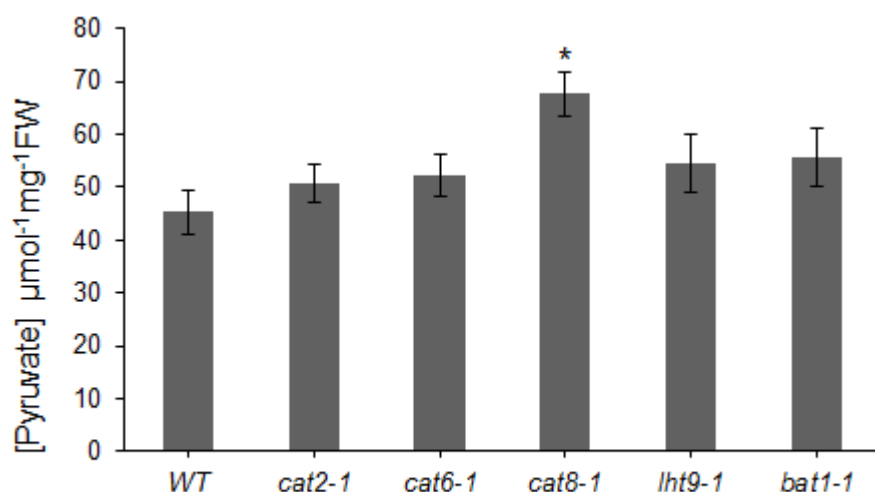


Figure 5.5. Measuring total pyruvate spectrophotometrically.

Wild-type and mutant seeds were germinated on $\frac{1}{2}$ MS plates, grown for 11 days then transplanted into sand. Plants were watered every second day with N-free Hoagland's solution plus 1 mM NH_4NO_3 and 1 mM alanine. Roots were harvested at 27 days after sowing and pyruvate was extracted to use in a pyruvate oxidase-coupled assay. Data are the mean of $n = 6 \pm \text{SEM}$. Values significantly different from wild-type at $p < 0.05$ are indicated by * (t-test).

The pyruvate oxidase assay revealed that only the alanine-insensitive mutant *cat8-1* had a significant increase in pyruvate levels in comparison to wild-type levels. No other changes in pyruvate levels were observed in any of the other mutants.

LC-MS was used to complement the GC-MS analysis by analysing a wide range of other metabolites. Standard compounds were used to identify a select number of metabolites (Table 5.2), which were then statistically analysed using t-tests. In a separate analysis, an untargeted approach was used to detect 6150 non-annotated compounds within the mutant and wild-type samples (Figure 5.6). This allowed a larger set of compounds to be tested to see whether a metabolic signal could be identified within the alanine-insensitive mutants.

Table 5.2 LC-MS metabolite analysis relative to wild-type

Experimental conditions were kept the same as for GC-MS analysis. Data are the mean of $n = 6 \pm$ SEM. Values significantly different using t-tests ($p < 0.05$) and correcting for false discovery rates using a q-value of 3% are shown in bold.

	Percentage metabolite abundance relative to WT $\pm$ SEM				
	<i>bat1-1</i>	<i>cat2-1</i>	<i>cat6-1</i>	<i>cat8-1</i>	<i>lht9-1</i>
2-Hydroxyglutarate	133.53 $\pm$ 16.72	215.90 $\pm$ 0.67	116.09 $\pm$ 3.69	109.51 $\pm$ 1.52	105.01 $\pm$ 4.37
2OG	62.12 $\pm$ 0.18	181.63 $\pm$ 2.96	87.45 $\pm$ 5.72	129.93 $\pm$ 3.80	68.13 $\pm$ 0.30
2Phosphoglycerate	93.40 $\pm$ 7.74	172.68 $\pm$ 1.47	46.81 $\pm$ 0.16	65.38 $\pm$ 1.51	66.16 $\pm$ 3.30
5P-DiRibose-1diP	119.87 $\pm$ 3.69	281.25 $\pm$ 3.98	69.12 $\pm$ 0.95	194.95 $\pm$ 3.90	66.70 $\pm$ 0.42
Adenine	101.22 $\pm$ 16.07	186.22 $\pm$ 0.90	101.01 $\pm$ 1.33	102.10 $\pm$ 1.64	96.27 $\pm$ 1.52
ADP	102.05 $\pm$ 9.86	130.31 $\pm$ 1.39	56.28 $\pm$ 0.89	51.31 $\pm$ 1.40	64.23 $\pm$ 1.11
ATP	121.21 $\pm$ 6.01	219.60 $\pm$ 1.64	76.82 $\pm$ 0.49	76.91 $\pm$ 1.75	115.13 $\pm$ 0.53
cis-aconitate	152.96 $\pm$ 13.30	176.16 $\pm$ 3.71	86.75 $\pm$ 7.85	102.15 $\pm$ 8.41	70.81 $\pm$ 15.76
Citrate	74.25 $\pm$ 11.10	171.75 $\pm$ 1.45	100.41 $\pm$ 3.56	64.46 $\pm$ 0.91	56.93 $\pm$ 3.77
Cytidine	98.10 $\pm$ 9.29	120.63 $\pm$ 7.05	86.17 $\pm$ 13.11	70.83 $\pm$ 5.52	46.56 $\pm$ 5.81
Deoxyuridine	53.00 $\pm$ 5.46	99.92 $\pm$ 3.63	53.97 $\pm$ 3.18	24.11 $\pm$ 1.95	22.99 $\pm$ 3.56
Fructose-1-6-Bisphosphate	90.99 $\pm$ 10.36	220.48 $\pm$ 4.37	60.52 $\pm$ 3.48	102.20 $\pm$ 3.34	62.06 $\pm$ 9.81
Fructose-6-phosphate	56.77 $\pm$ 12.06	146.15 $\pm$ 3.18	45.23 $\pm$ 1.42	39.23 $\pm$ 2.01	36.61 $\pm$ 2.36
Fumarate	108.64 $\pm$ 0.09	63.98 $\pm$ 0.93	191.99 $\pm$ 5.24	159.53 $\pm$ 6.07	153.62 $\pm$ 11.37
Glucose	64.70 $\pm$ 11.20	127.23 $\pm$ 4.89	237.84 $\pm$ 5.18	56.88 $\pm$ 2.57	41.67 $\pm$ 9.68
Glucose-1-Phosphate	75.58 $\pm$ 12.55	121.60 $\pm$ 0.70	69.89 $\pm$ 0.93	45.99 $\pm$ 1.39	53.60 $\pm$ 1.36

	Percentage metabolite abundance relative to WT $\pm$ SEM				
	<i>bat1-1</i>	<i>cat2-1</i>	<i>cat6-1</i>	<i>cat8-1</i>	<i>lht9-1</i>
Glucose-6-phosphate	56.77 $\pm$ 12.06	146.16 $\pm$ 3.18	45.23 $\pm$ 1.42	39.23 $\pm$ 2.01	36.61 $\pm$ 2.36
Glutathione	33.27 $\pm$ 12.33	200.23 $\pm$ 7.83	98.67 $\pm$ 4.00	88.37 $\pm$ 3.64	57.14 $\pm$ 3.94
Guanosine	77.42 $\pm$ 12.49	199.53 $\pm$ 4.44	153.85 $\pm$ 8.03	57.58 $\pm$ 2.18	54.15 $\pm$ 3.72
Lactate	83.53 $\pm$ 17.29	228.89 $\pm$ 0.91	72.89 $\pm$ 0.50	79.46 $\pm$ 2.96	45.46 $\pm$ 2.52
Malate	82.34 $\pm$ 15.25	138.22 $\pm$ 1.31	111.72 $\pm$ 3.29	74.89 $\pm$ 0.44	76.30 $\pm$ 1.84
NAD ⁺	85.09 $\pm$ 13.24	116.19 $\pm$ 4.00	45.58 $\pm$ 1.31	52.48 $\pm$ 1.77	62.63 $\pm$ 5.55
Phosphoenolpyruvate	73.80 $\pm$ 2.04	178.63 $\pm$ 3.50	83.17 $\pm$ 0.40	80.39 $\pm$ 2.16	46.04 $\pm$ 2.45
Pyruvate	39.80 $\pm$ 14.93	163.98 $\pm$ 3.64	60.37 $\pm$ 9.71	71.01 $\pm$ 1.73	44.48 $\pm$ 5.39
Ribose-5-Phosphate	96.31 $\pm$ 14.88	207.91 $\pm$ 3.40	98.45 $\pm$ 6.26	89.59 $\pm$ 3.23	70.98 $\pm$ 6.09
Succinate	106.35 $\pm$ 14.38	174.11 $\pm$ 0.40	89.49 $\pm$ 3.01	94.92 $\pm$ 1.63	70.57 $\pm$ 2.50
Thymine	30.18 $\pm$ 1.18	33.49 $\pm$ 21.38	33.59 $\pm$ 0.96	53.93 $\pm$ 32.08	37.85 $\pm$ 10.06
Uracil	96.72 $\pm$ 7.27	130.29 $\pm$ 16.91	83.84 $\pm$ 16.34	53.92 $\pm$ 11.10	35.99 $\pm$ 43.81
Uridine	116.39 $\pm$ 17.49	146.55 $\pm$ 0.78	176.25 $\pm$ 4.19	98.19 $\pm$ 0.43	109.05 $\pm$ 1.37

A metabolic signature common to the alanine-insensitive mutants was not identified among the LC-MS identified metabolites (Table 5.2). The alanine-insensitive mutant, *cat2-1*, showed the most metabolite changes, with 19 out of 29 identified metabolites showing a significant increase in abundance in comparison to wild-type levels. No other obvious patterns could be drawn from the data, and perhaps all other significant changes are the result of perturbations

due to the loss-of-function of an amino acid transporter within the mutants, but cannot be specifically linked to the alanine insensitive phenotype because they do not occur in all four alanine-insensitive mutant lines, or also occur in the *bat1-1* negative control line.

The data obtained via LC-MS analysis of 6150 unidentified compounds in mutant and wild-type plants was used in a principal component analysis to test for variances between the genotypes. If a common metabolic signature existed within the alanine-insensitive mutants, I would expect to see those mutants group together separately from wild-type and the alanine-sensitive mutant *bat1-1*. This pattern was not observed, meaning that a metabolic signature was not identified in this analysis. However, because the prospective difference in metabolite levels that produce the metabolic signal are potentially quite small, the large dataset might be masking these differences. For this reason, I filtered the metabolite data to isolate only metabolites that were significantly different at $p < 0.05$ (ANOVA) and metabolites that were observed to have a 10-fold difference between the lowest detected abundance and the highest detected abundance (Figure 5.7). I reasoned that applying these filters would reduce the dataset to those metabolites most likely to be detected as a metabolic signal. Again, however, the alanine-insensitive lines did not group together indicating that they do not share a common variance, such as a metabolic signature (Figure 5.7).

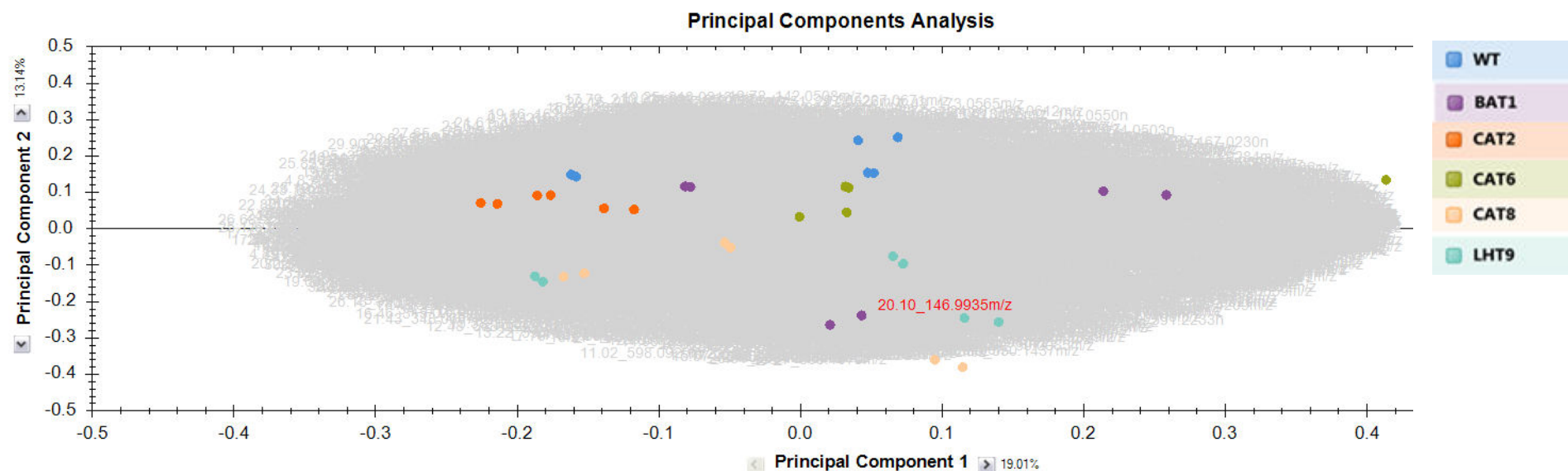


Figure 5.6 Principal component analysis of LC-MS data.

Untargeted analysis of LC-MS data identified 6150 compounds. These compounds were used in a principal component analysis to assess whether variables within genotypes group together.

It was also important to investigate further the alanine-insensitive mutant response to exogenous alanine. We know from chapter 3 that supplying exogenous alanine to wild-type roots results in an intracellular accumulation of alanine. I therefore wanted to know whether alanine also accumulates in the mutants, and whether the level of alanine accumulation differed.

I used GC-MS to quantify soluble alanine in alanine-insensitive mutant root and shoot methanol extracts. I included wild-type and an alanine-sensitive mutant *bat1-1* as negative controls (Figure 5.8). In shoot tissues, in the absence of exogenous alanine, *cat2-1* and *cat8-1* have significantly less alanine than the wild-type under the same condition (Figure 5.8 A). When exogenous alanine is present in the media, *bat1-1*, *cat6-1* and *lht9-1* accumulate significantly more alanine in shoot tissues than wild-type. It is surprising that *bat1-1* accumulates shoot alanine as this alanine-sensitive mutant has been used as a negative control due to previously having no growth phenotype. However, *bat1-1* is an amino acid transporter mutant and therefore variation in amino acid levels may be expected. In root tissues, *lht9-1* accumulated significantly more alanine than wild-type in the absence of exogenous alanine (Figure 5.8 B). No other significant differences in alanine concentration were found in root tissues. This is likely due to the large variation observed in the mean from the 6 biological replicates.

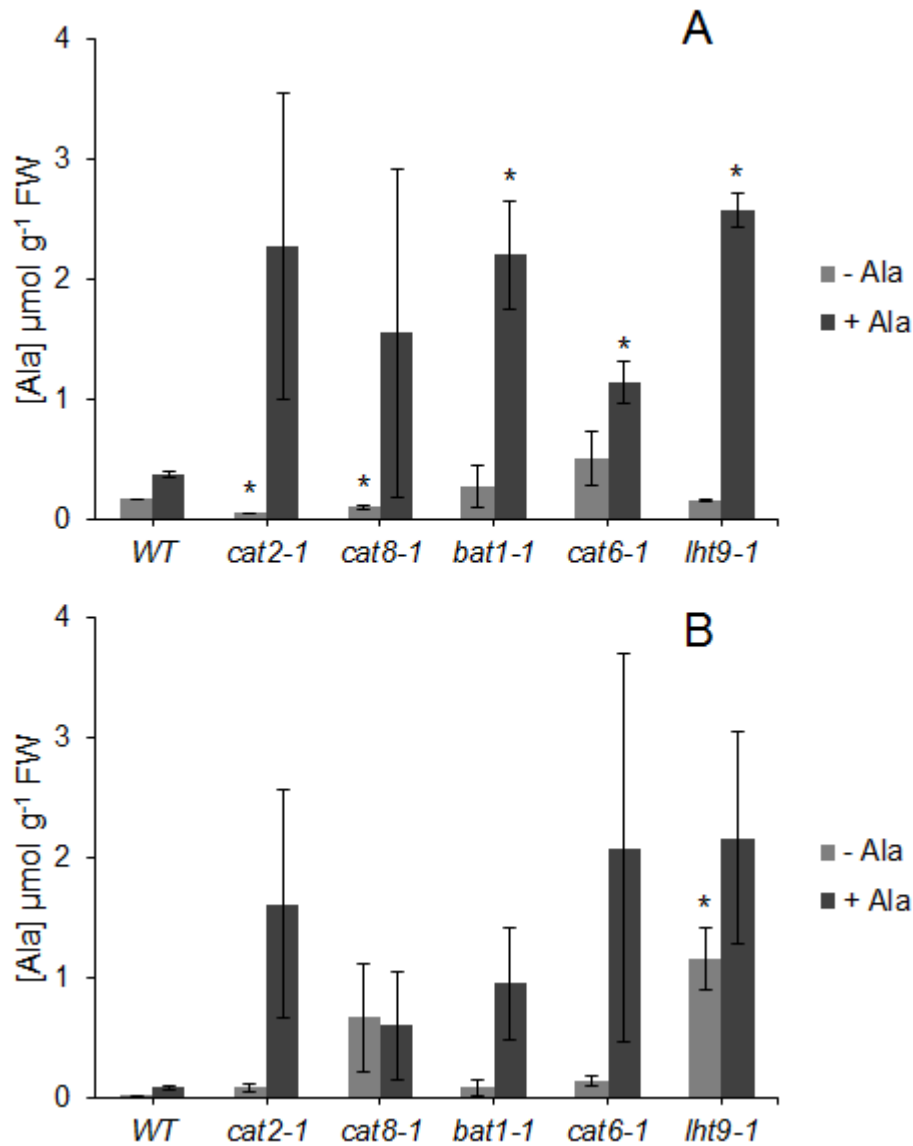


Figure 5.8. GC-MS quantification of soluble alanine in the roots and shoots of alanine insensitive mutants and wild-type.

Sand-grown plants fed either 1 mM NH_4NO_3 or 1 mM alanine + 1 mM NH_4NO_3 were sampled at 23 days after sowing for metabolite extraction. Metabolite extractions were derivitized then run on a GC-MS. Shoots (A); and Roots (B). Data are the mean of $n=6 \pm \text{SEM}$. Alanine insensitive mutants significantly different from wild-type under the same condition at $p<0.05$ are indicated by * (t-tests).

5.2.5 A comparison of nitrate uptake in both the alanine-insensitive mutant lines and wild-type, in the presence and absence of exogenous alanine

Previous work has established that exogenously supplied amino acids reduce nitrate uptake (Lee et al., 1992; Muller, 1992; Muller et al., 1995). We also know that this is accompanied by a reduction in transcripts relating to nitrate uptake (Chapter 3, Figure 3.10) (Muller et al., 1995; Nazoa et al., 2003). In the alanine-insensitive mutants, however, an increase in nitrate transporter transcripts is observed (Figure 5.4), which suggests that this may be accompanied by a derepression of nitrate uptake. I conducted a simple labelled nitrate feeding experiment to determine whether this was the case.

One of the alanine insensitive mutants, *cat2-1*, was selected as a representative for the four alanine insensitive mutants, along with wild-type for the experiment. Both wild-type and *cat2-1* were supplied with 0.5 mM K¹⁵NO₃ + 0.5 mM (NH₄)₂SO₄ (for minus alanine treatment), or 0.5 mM K¹⁵NO₃ + 0.5 mM (NH₄)₂SO₄, and 1 mM alanine (for plus alanine treatment) (Figure 5.9).

The data obtained for wild-type corroborates (same as in Figure 3.9) other published data that nitrate uptake is inhibited when amino acids are exogenously supplied (Lee et al., 1992; Muller, 1992; Muller et al., 1995) (Figure 5.9 A). In contrast however, nitrate uptake is unaffected by the presence of exogenous alanine in the *cat2-1* mutant (Figure 5.9 B).

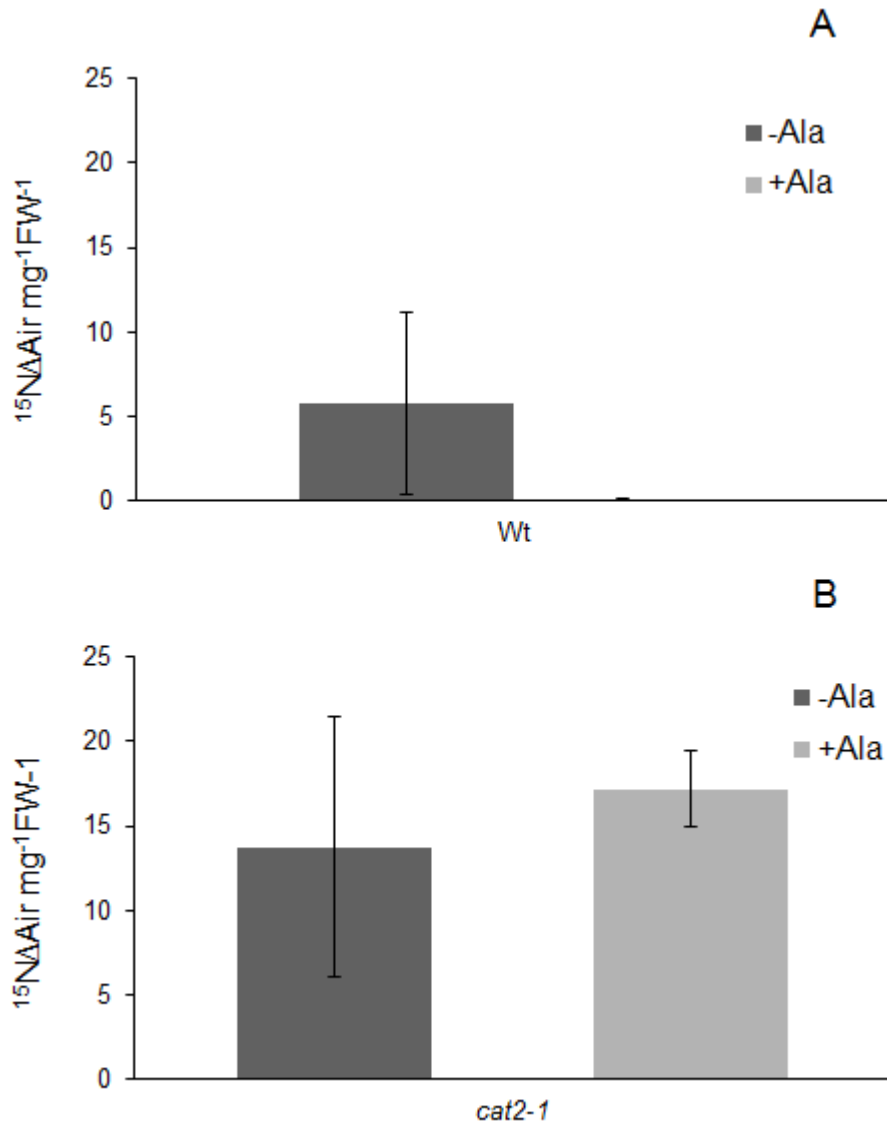


Figure 5.9. K^{15}NO_3 uptake in alanine insensitive mutants and WT.

Arabidopsis wild-type and *cat2-1* seeds were sterilised and sown on to plates containing $\frac{1}{2}$ MS. After 11 days, seedlings were transplanted to flasks containing 0.1 mM CaCl_2 for a 24 hr starvation period. After 24 hours, wild-type seedlings (A), and *cat2-1* seedlings (B), were fed either 0.5 mM K^{15}NO_3 + 0.5 mM $(\text{NH}_4)_2\text{SO}_4$ (-Ala), or 0.5 mM K^{15}NO_3 + 0.5 mM $(\text{NH}_4)_2\text{SO}_4$, and 1 mM alanine. Seedlings were sampled after 5 minutes, freeze-dried, and the quantity of ^{15}N was analysed using isotope-ratio mass spectrometry. Data are the mean of $n=3 \pm \text{SEM}$. T-tests were performed for -Ala against +Ala but none of the data were significant.

5.2.6 Characterisation of the growth phenotype of alanine insensitive mutants and wild-type

Thus far in this project, I have focused on rosette size to detect growth phenotypes that differ between mutant lines and wild-type. Other developmental and/or organ-specific growth phenotypes may be observed in the alanine-insensitive mutants. I therefore conducted a series of in-depth growth analyses in order to ascertain any differences.

Firstly, I recorded the dry weight of whole seedlings at 11 days after sowing, directly after they had been removed from the agar plate (Figure 5.10).

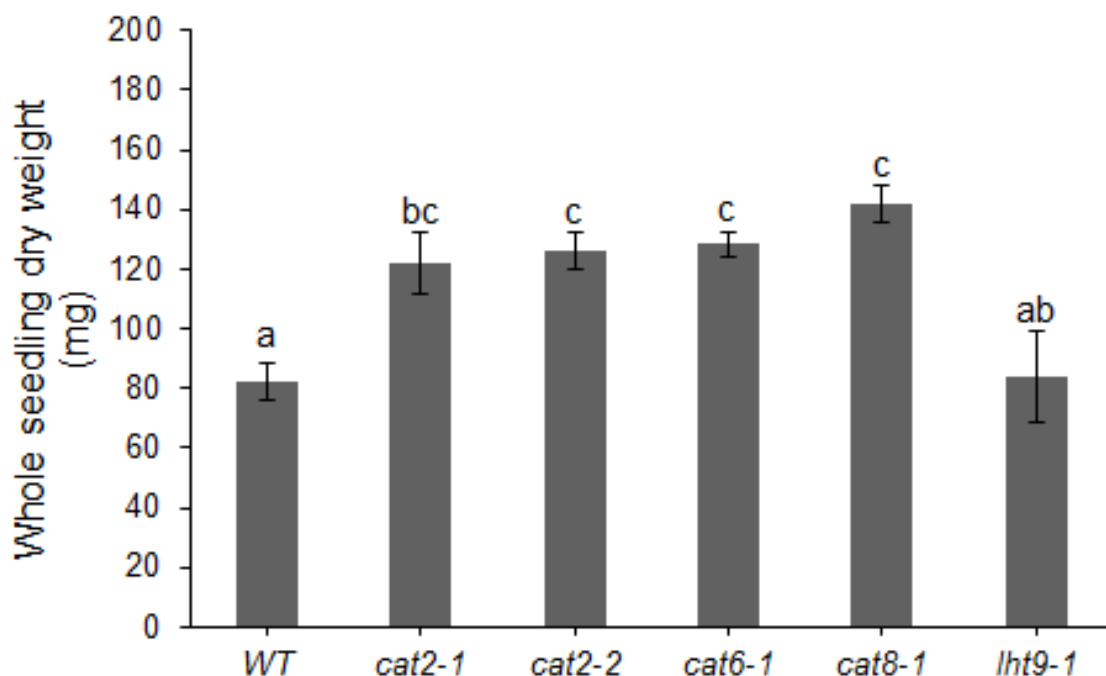


Figure 5.10. Comparing whole seedling mass of wild-type and alanine-insensitive mutants.

Wild-type and mutant seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, 30 seedlings per genotype were sampled, dried and weighed. Data are the mean of $n=30 \pm$ SEM. Genotype seedling dry weights significantly different from each other are indicated by letters (One-way ANOVA).

All mutant lines, except *lht9-1* had greater biomass than wild-type at 11 days after sowing (Figure 5.10).

Plants grown on plates receive an extra carbon source in the form of 0.8% (w/v) sucrose. Nitrogen and carbon metabolism are intimately linked and both are required for growth. It is possible that the mutants are able to accumulate more nitrogen than wild-type, due to the ability to avoid the feedback inhibition of nitrate uptake. This increase in nitrogen supply would need an equal increase in carbon supply in order for the excess nitrogen to be utilised for amino acids synthesis and contribute towards new growth. Mutants grown on plates, with an extra carbon source, are significantly larger than wild-type, which supports the idea that carbon is the limiting growth factor in the alanine-insensitive mutants. I therefore wanted to further test whether supplying plants with a greater carbon source, in the form of high light treatment, would produce mutants with larger vegetative growth than wild-type.

Selecting the optimum level of light to avoid light-stress was paramount for this experiment. Any additional stresses on the plant could have altered the growth and thus masked any effects of increased carbon availability. In the literature, high light treatment with the intent on causing light-stress in *Arabidopsis* is reported to occur at around 1000 μE (Rossel et al., 2002; Giacomelli et al., 2006). Standard growth conditions for *Arabidopsis* often recommend growing plants at 145 μE , which is the light intensity we most frequently use in our laboratory and is of low intensity. Also taken in to consideration were curves produced by varying the level of light intensity and measuring the rate of CO_2 consumption in wild-type *Arabidopsis* (Eckardt, 1997). This revealed that photosynthetic rate begins to plateau at approximately 600 μE , meaning that if the intention is to increase carbon availability; increasing light intensity beyond this point would be futile. I therefore decided to use two levels of light intensity to test whether increased carbon availability would increase rosette growth in the alanine insensitive mutants: a medium intensity of 400 μE and a higher intensity of 600 μE .

Soil grown wild-type, alanine-insensitive lines and two alanine-sensitive lines (*aap2-1* and *bat1-1*) were grown under two high light regimes; one with an intensity of 400 μE (Figure 5.11 A), and one with an intensity of 600 μE (Figure 5.11 B). At 400 μE mutant rosette size

was not observed to be significantly larger than wild-type rosette size. The alanine-sensitive mutant line *bat1-1*, had a rosette that was significantly smaller than wild-type (Figure 5.11 A). At the higher light intensity of 600 μ E, mutant line rosettes were generally smaller than wild-type, indicating that this level of light possible induced light stress in these lines.

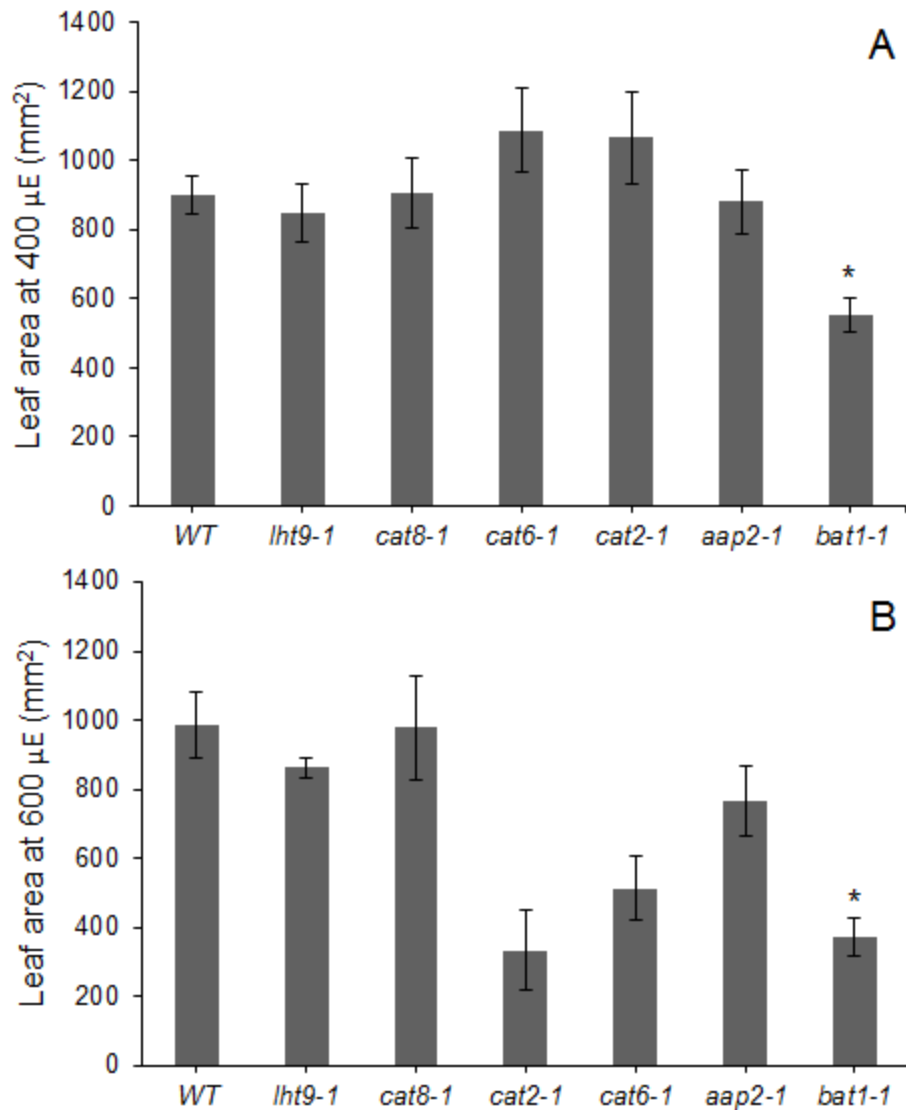


Figure 5.11. Comparing rosette size of wild-type and alanine insensitive mutants grown under high light treatment.

Wild-type and mutant seeds were germinated on moist filter paper, then transplanted on to standard soil with vermiculite. Plants were photographed 36 days after sowing and leaf area was calculated using Leaf Lab software. Plants were grown under light intensity of 400 μE (A) and Plants were grown under a light intensity of 600 μE (B). Data are the mean of $n=7 \pm$ SEM. Values significantly different from wild-type at $P \leq 0.05$ (t-test) indicated by *.

There are a number of critical points in the development of Arabidopsis, including; germination, cotyledon development, primary root development, rosette leaf development, inflorescence emergence, silique ripening and senescence (Boyes et al., 2001). To understand whether any other developmental differences occurred between mutants and

wild-type, I recorded; bolting time, appearance of first flowers, the beginning of senescence, bolt height and finally seed mass for each individual plant (Figure 5.12).

In the absence of alanine, *cat6-1* bolted significantly later than wild-type (Figure 5.12 A). However, in the presence of alanine, *cat2-1* and *cat6-1* bolted later, whereas *cat8-1* bolted significantly sooner than wild-type (Figure 5.12 B). The time taken for the first flowers to appear in *cat2-1* and *cat6-1* was significantly longer than in wild-type in the presence of alanine (Figure 5.12 D). No differences were observed in flowering time in the absence of alanine (Figure 5.12 C). *cat2-2*, *cat6-1* and *lht9-1* senesced earlier than wild-type in the absence of alanine (Figure 5.12 E). However, when alanine was exogenously supplied, *cat6-1* senesced much later than wild-type (Figure 5.12 F). Bolts were significantly shorter than wild-type in *cat2-2* and *cat8-1* (Figure 5.12 G), whereas, when alanine was supplied, *cat2-1*, *cat8-1* and *lht9-1* displayed shorter bolts than wild-type (Figure 5.12 H). All lines except *cat2-2* recorded a lower total seed mass than wild-type (Figure 5.12 I). Yet, on supply of alanine, *cat2-1* and *cat6-1* were observed to produce a significantly higher seed mass than wild-type. This was in contrast to *cat8-1*, and *lht9-1*, both of which produced a significantly lower seed mass.

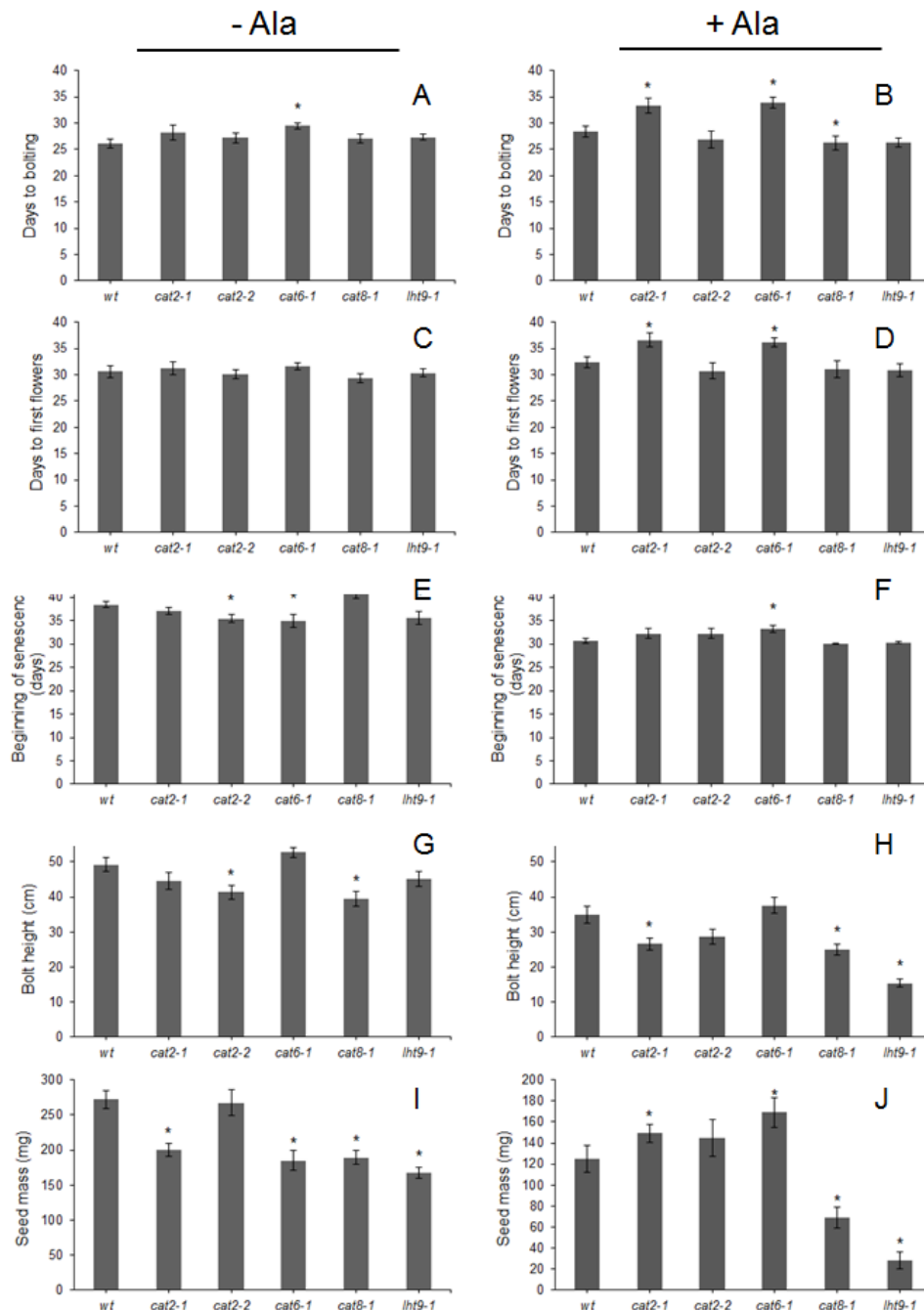


Figure 5.12. A comparison of various stages of development between wild-type and alanine insensitive mutants

Wild-type and mutant seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus either 1 mM NH_4NO_3 (- Ala) or 1 mM NH_4NO_3 + 1 mM alanine (+ Ala). A + B) compares number of days to bolting; C + D) compares bolt height; E + F) compares the number of days to the appearance of the first flowers; G + H) compares the start of senescence; and I + J) compares seed mass. Data are the mean of $n = 12 \pm \text{SEM}$. Values significantly different to wild-type at $p < 0.05$ are indicated by * (t-test).

To complete the growth analysis, I measured the shoot and root biomass of wild-type and alanine-insensitive mutants (Figure 5.13).

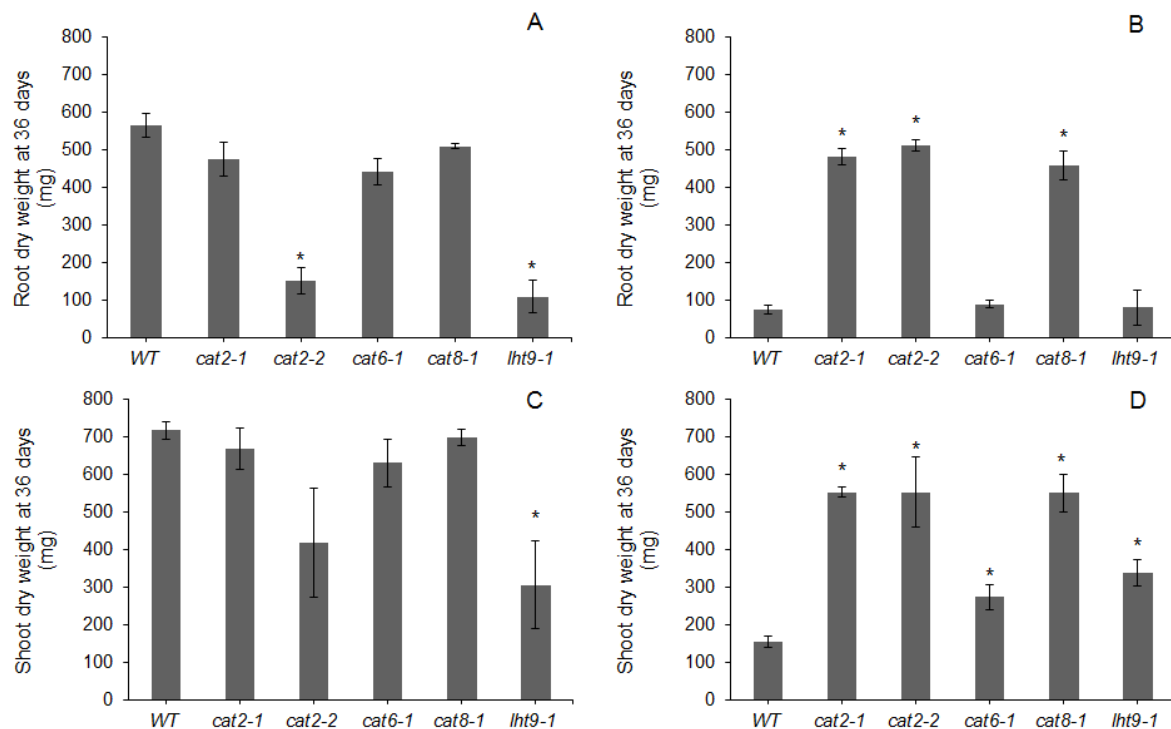


Figure 5.13. Comparing root and shoot biomasses in alanine and non-alanine fed wild-type and alanine insensitive mutants.

Wild-type and mutant seed was germinated on ½ MS plates for 11 days, and then transplanted in to a sand and vermiculite soilless mixture. Trays were watered every second day with 400 ml N-free Hoagland's solution, + either 1 mM NH_4NO_3 (- Ala) or 1 mM NH_4NO_3 + 1 mM alanine (+ Ala). At day 36 after sowing, plants were harvested, dried and weighed. Root dry weight was recorded for plants fed - Ala (A), and + Ala (B). Shoot dry weight was recorded from plants fed - Ala (C) and + Ala (D). Data are the mean of $n = 3 \pm \text{SEM}$. Values significantly different to wild-type at $p < 0.05$ are indicated by * (t-test).

As expected, shoot biomass was greater in all of the alanine-insensitive mutants than in wild-type when alanine was supplied (Figure 5.13 D), which corroborates with the increased leaf area observed in the growth screen in Chapter 4. In comparison to wild-type, root biomass was decreased significantly in *cat2-2*, which is a second independent T-DNA insertion in the CAT2 gene, and *lht9-1* in the absence of exogenously alanine (Figure 5.13

A). However, when alanine was provided, root biomass increases were observed in *cat2-1*, *cat2-2* and *cat8-1* in comparison to wild-type (Figure 5.13 B).

5.3 Discussion

5.3.1 Alanine-insensitive mutants are potentially perturbed in nitrogen-status sensing

Alanine-insensitive mutants are able to take up alanine at normal rates (Figure 5.3). Such a result indicates that the alanine insensitivity phenotype is not a result of impaired uptake of exogenous alanine, but is likely due to effects of the mutations on metabolite abundances, probably in specific tissues or cell compartments. Alterations in metabolite abundances may affect nitrogen-status signalling. The likelihood of a signalling role was further supported by measuring nitrate transporter transcripts. I observed an induction of *NRT1.1*, *NRT2.1* and *NRT2.2* transcripts under alanine treatment (Figure 5.4). Ordinarily, in wild-type plants, supplying exogenous alanine causes alanine to accumulate within the plant; which activates the feedback inhibition of nitrate transporter transcripts, resulting in reduced nitrate uptake. In alanine-insensitive mutants, down-regulation of nitrate transporter transcripts is absent, possibly meaning that increased alanine levels has not been detected. Additional support for this explanation comes from the fact that nitrate uptake is unaffected in the *cat2-1* mutant when exogenous alanine is provided (Figure 5.9). Taken together, these results may indicate that nitrate-status sensing has been perturbed. Alternatively, the T-DNA insertion mutation in the amino acid transporter mutants could affect metabolite abundances within the plant, either in specific tissues or cell compartments. This would lead to a redistribution of metabolites at a subcellular level, which could potentially be sensed downstream by the nitrogen-status signalling pathway, resulting in the observed phenotype.

5.3.2 Some alanine insensitive mutants have detectable transcripts

An added layer of complexity is that two of the alanine-insensitive mutants have detectable transcripts (Figure 5.2 C, D). A reduced level of transcript is detected in *cat6-1*, indicating that this mutant is likely to be a knockdown rather than a knockout, and an increased level of transcript is found in *cat8-1*, suggesting this transcript may be overexpressed. It is not uncommon for overexpression to result in the same phenotype as a loss-of-function mutant. This is because the act of overexpression can interfere with the function of the protein or the complex (Prelich, 2012). A good example of this occurs in *Saccharomyces cerevisiae* where overexpression of histone pairs, *SPT5*, *SPT6* or *SPT16* each causes the same transcription-related phenotypes as loss-of-function mutations in those genes (Clark-Adams and Winston, 1987; Clark-Adams et al., 1988; Malone et al., 1991; Swanson et al., 1991). These genes all encode parts of a multiprotein complex, which suggests that overexpression interferes with the correct formation of this complex. Interestingly, in the case of the histones, overexpressing each gene restores the wild-type phenotype, suggesting that re-establishing stoichiometry is able to correct the disruption in the formation of the protein complex. It is therefore possible that the four alanine-insensitive mutants function as a multiprotein complex, and that altering the stoichiometry by loss-of-function, knockout or knockdown mutants all disrupt the formation of the complex, resulting in the same phenotype. Alternatively, if no complex is formed, overexpression may disrupt the protein function resulting in a loss-of-function mutant and a knockdown mutant might result in significantly diminished functioning protein, thus appearing to produce the same phenotype as the loss-of-function mutants.

5.3.3 The search for a metabolic signal

I hypothesised that a metabolic signature, common to all the alanine-insensitive mutants might exist, which causes an indirect perturbation in nitrogen signalling due to being sensed by an as yet unknown mechanism. Metabolic profiling of alanine-insensitive mutants, wild-

type and the negative control *bat1-1*, and subsequent statistical analysis revealed that there were three metabolites that were significantly different in the alanine-insensitive mutants but not in *bat1-1* (Table 5.1). However, not all metabolites were detected using the GC-MS method, therefore LC-MS was also used to detect a broader range of metabolites. Despite detecting 6150 compounds using the LC-MS method (Figure 5.6), I was unable to identify a common metabolic signature within the alanine-insensitive mutants. There is the possibility that the change in metabolite levels of the corresponding metabolic signature was too small to affect the variance in a principal component analysis. An alternative possibility, however, is that the metabolite signal occurs in a specific subcellular compartment. Metabolite levels are altered within a compartment in the cell, for example, the cytoplasm, which is sensed by the nitrogen-status signalling pathway to cause the observed signalling perturbations. Since only total metabolite levels are detected using both GC-MS and LC-MS, differences in subcellular metabolite levels could be masked and thus it remains possible that a metabolic signature does exist but that the methods used in this thesis were not sensitive enough to detect one.

Statistical analysis of the GC-MS metabolite profile data identified three metabolites that were significantly increased in the alanine-insensitive mutants, but not in wild-type or the negative control *bat1-1*. These metabolites were mevalonic acid, threonine and ornithine (Table 5.1). Mevalonic acid is an intermediate in the cytosolic mevalonate pathway (MVA), which synthesises the building blocks for isoprenoid biosynthesis. There are two independent pathways for isoprenoid biosynthesis: the cytosolic mevalonate pathway for synthesis of sterols, sesquiterpenes, triterpenoids; and the plastidic non-mevalonate pathway for the synthesis of plastidic isoprenoids, such as carotenoids, phytol, and diterpenes (Lichtenthaler et al., 1997; Rohmer, 1999). Both pathways produce isopentenyl diphosphate (IPP), from which all other isoprenoids are formed. It is unclear why the concentration of a metabolite in the mevalonate pathway is increased in the alanine-insensitive mutants, or whether this increase translates to increased production of

isoprenoids. Sterols have a role in the fluidity and permeability of membranes since free hydroxyl groups in the molecules enable its specific interaction with phospholipids and proteins in membranes (Piironen et al., 2000), therefore sterols can regulate membrane structure. Plant sterols are also involved in the formation of lipid “rafts” within membranes which has been shown to decrease temperature sensitivity thus allowing biological processes to take place at a wider range of temperatures (Beck et al., 2007; Dufourc, 2008). Accordingly, it is possible that the alanine-insensitive mutants are less sensitive to temperature variations which is something that could be investigated in the future characterisation of these mutants.

Threose is a 4-carbon sugar and a diastereomer of erythrose, which is used in lignin and anthocyanin synthesis. Lignins are cell wall components that contribute to their rigidity (Barcelo, 1997) and anthocyanins are pigments thought to provide a defence against photo-oxidative damage caused by light stress (Steyn et al., 2002). Threose accumulates in alanine-insensitive mutants suggesting that the perturbation in nitrogen signalling may have a secondary effect of increasing cell wall rigidity and protecting against light stress. Lignin and anthocyanin concentrations were not measured; however, there was no obvious accumulation of anthocyanins in the mutants based on the lack of discolouration of the leaves. Lignin and anthocyanin levels would need to be measured to determine whether they are elevated in response to increased threose levels.

Ornithine is synthesised from glutamate and is a required intermediate for arginine biosynthesis in plants (Shargool et al., 1988). Additionally, it is utilised in the biosynthesis of alkaloids and polyamides. Arginine is an important amino acid as it can serve as a nitrogen store due to its high nitrogen content (Winter et al., 2015). Alanine-insensitive mutants accumulate ornithine, which may be in response to accumulating nitrogen, and therefore generating a need to store the excess nitrogen. Storing excess nitrogen in the form of arginine would be a viable option for the plant, as this could be used later if nitrogen supplies dwindled. However, arginine does not appear to accumulate in these mutants. Alternatively,

the ornithine accumulation could be a result of glutamate accumulation as a direct result of the alanine aminotransferase reaction in response to increased alanine levels. Glutamate levels appear to remain consistent and may be regulated, therefore, excess glutamate may be used to synthesise ornithine, which does not appear to be regulated, in order to regulate glutamate levels.

5.3.4 Alanine-insensitive mutants develop differently to wild-type

Some of the alanine-insensitive mutants displayed signs of having increased nitrogen-use efficiency. For example, *cat2-1* had delayed bolting and flowering times (Figures 5.12 B, D) and had higher seed mass (Figure 5.12 J) and shoot biomass (5.13 D) than wild-type when exogenous alanine was supplied. Bolting and flowering are critical points in Arabidopsis development as this signifies the end of shoot and root growth and the start of reproductive organ growth (Ikram et al., 2012). Post bolting and flowering, nitrate uptake is dramatically reduced and nitrogen resources from the leaves are translocated to the reproductive organs (Cohen, 1971). A mathematical model that encapsulates previous experimental observations and measurements was used to predict that flowering time is intrinsically linked to nitrogen availability. The model predicts that increased availability of nitrogen will delay flowering and decreased nitrogen availability will cause early flowering (Guilbaud et al., 2015). This is supported by experimental data that observed nitrogen limited plants flower earlier (Paul-Victor et al., 2010; Züst et al., 2012). One explanation for this occurrence is the ‘peak N’ theory (Guilbaud et al., 2015), which states that there is a theoretical upper limit to flowering time that will occur at the time at which the whole plant nitrogen uptake, and hence the whole plant growth rate are highest. If more nitrogen is available, ‘peak N’ will be delayed because it will take longer for nitrogen uptake and plant growth rate to reach a maximum. In *cat2-1* and *cat6-1* under alanine treatment, it appears that flowering is delayed, possibly because the mutants have a perceived increase in availability of nitrogen. We know that nitrate uptake is not inhibited under alanine treatment and that alanine is taken up at normal

rates, therefore it is possible that the additional internal availability of nitrogen is acting to delay flowering in order to maximise available resources.

The fact that seed yield is increased in *cat2-1* and *cat6-1* and that shoot biomass is increased in all the alanine-insensitive mutants is evidence that the plants are more nitrogen-use efficient than wild-type when exogenous alanine is provided. Mutants and wild-type have exactly the same nitrogen availability, yet the mutants are able to take up more nitrogen for use in the growth of vegetative tissues and seed yields.

5.4 Summary

Alanine-insensitive mutants are unaffected in alanine uptake rates, yet have an increased growth phenotype and an increase in the abundance of *NRT1.1* transcript was observed, accompanied by an absence of nitrate uptake inhibition. This potentially indicates that these mutants are perturbed in nitrogen-status signalling as a result of the mutation altering metabolite levels, which then feed in to the signalling pathway. A metabolic signal causing this perturbation was not identified, but it remains possible that one exists as not all metabolites were quantified and variation in subcellular metabolite levels was not taken in to consideration. Alternatively, changes in metabolite levels caused by the mutation may be causing these changes without interacting with the nitrogen-status signalling pathway, something that could be investigated further in the future.

Further characterisation of the alanine-insensitive mutants revealed that they might be more nitrogen-use efficient. Nitrate uptake is unaffected by alanine treatment, which possible leads to a higher total nitrogen content. Further to this, flowering is delayed in some mutant lines that might allow for a longer period of nitrogen acquisition, contributing to the increased seed yield also observed in some of the alanine-insensitive mutants.

Chapter 6: Further characterisation of the role of CAT2 in amino acid insensitivity.

6.1 Introduction

The aim of the work described in this chapter was to further develop our understanding of the role of CAT2 in amino acid insensitivity. One of the experimental goals was to test whether the amino acid insensitivity observed in the *cat2-1* mutant can be unequivocally attributed to the loss-of-function of the CAT2 transporter. This will be achieved by testing a second, independent T-DNA mutant. This chapter further aims to discover whether other amino acids, apart from alanine, can lead to amino acid insensitivity and whether these amino acids need to be metabolised for the effect to be observed. Additionally, to understand which substrates may be transported by the CAT2 protein, a microscale thermophoresis binding assay will be used to identify binding substrates. The final aim of this chapter was to identify proteins that interact with the CAT2 transporter using a pull-down assay to identify additional components of the signalling pathway.

6.1.1 Cationic amino acid transporter 2

The work in this chapter focuses on CAT2 and the corresponding knockout mutant *cat2-1*. It was only feasible to select one of the four alanine-insensitive mutants to characterise further within the scope of this project.

CAT2 is a member of the cationic amino acid transporter (CAT) family, which are part of the amino acid polyamine choline-type (APC-type) family. CAT2 is predicted to have a 14 transmembrane topology and is localised to the tonoplast (Su et al., 2004). To date, no transport substrates have been identified for CAT2, despite uptake studies in yeast uptake mutants overexpressing CAT2 (Su et al., 2004) and *Xenopus* oocytes (Yang et al., 2014). This could indicate that the protein has developed a more specialised role outside of its transport capacity, such as a sensory role. However, it is important to note that the CAT2

protein is targeted to the tonoplast and therefore could not be reconstituted successfully into the oocyte plasma membrane; and not all amino acid substrates could be tested in the yeast assay. Thus opening up the distinct possibility that CAT2 does transport amino acids, but that these amino acids have not been identified yet.

To summarise, I will focus on CAT2 because this transport protein shares similarities with the *path* transceptor in animals. Namely, the fact that CAT2 is localised to the tonoplast, the environment of which is similar to the lysosome in animals, where *path* can be found (Oegmundsdottir et al., 2012). Yet, it is also tantalising that no transport substrates have been identified, leaving the possibility of a more specialised role outside of the transport capacity.

6.1.2 Alanine-insensitive amino acid transporters may form a functional signalling complex.

As discussed in the previous chapter, an unanticipated number of amino acid transporter mutants were observed to be insensitive to alanine. I developed two hypotheses in order to explain this unexpected result. The first hypothesis, which is discussed in detail in chapter 5, suggests that a perturbation in local metabolite levels, common to all four transporter mutants, is generated by the modification of transporter protein levels.

The second hypothesis suggests that the four transporter proteins could physically interact to form a functional complex, thus the removal of each protein in turn results in the same growth phenotype. Since CAT2, CAT6 and CAT8 are localised to root cell tonoplast and plasma membranes, it is possible that they physically interact to form a complex. There is currently no available data on the subcellular localisation of LHT9, but for the purpose of this hypothesis, one could assume that it would also be localised to root cell tonoplast and/or plasma membranes. CAT2 appears to be localised exclusively to the tonoplast as evidenced by transiently overexpressing CAT2 in tobacco and Arabidopsis protoplasts (Su et al., 2004; Yang et al., 2014). It can be extremely difficult to differentiate between tonoplast and plasma

membrane, however, in the CAT2 confocal images, the marked membrane is clearly bulging around the nucleus, thus indicating that it is tonoplast and not plasma membrane. The CAT6 transporter is likely to be targeted to the plasma membrane, as evidenced by YFP-CAT6 fusion proteins in Arabidopsis (Hammes et al., 2006). The published images are not overlaid with a membrane marker, which makes it difficult to determine for certain that the transporter localises to the plasma membrane. However, no transvacuolar strands are visible and the YFP marked membrane does not bulge around the nucleus, which suggests plasma membrane localisation. Overexpression of CAT8 in protoplasts (Su et al., 2004) and expressing CAT8 under the endogenous promoter in Arabidopsis (Yang et al., 2010) showed that CAT8 may localise to both tonoplast and plasma membrane. Based on the localisation patterns for these transporters, it may be possible for them to physically interact. CAT6 could be transported to the tonoplast via endocytosis which is reminiscent of PAT1, the human amino acid proton assisted transporter, which is mobilised to the lysosome via endocytosis, where it forms a complex called the nutrisome (Oegmundsdottir et al., 2012). The nutrisome recruits mTORC1 to the complex, which activates it to promote cell growth. If the process is similar in plants and a complex of signalling proteins is formed that includes the four amino acid transporter, this would explain why knockouts of each transporter result in the same growth phenotype.

6.2 Results

6.2.1 Investigation of a second independent T-DNA insertion in the *CAT2* gene to confirm that the phenotype observed in the *cat2-1* mutant is due to the disruption of the *CAT2* transporter.

T-DNA insertion mutants contain on average approximately 1.5 insertions per line (Alonso, 2003). This means that a phenotype associated with a T-DNA insert in a particular gene could in fact be due to an as yet undiscovered mutation elsewhere in the genome. There are several examples of the observed phenotypes in T-DNA mutants not being linked to the known T-DNA insertion (Vitha, 2003; Adham et al., 2005). Thus indicating that an untagged allele could be responsible for the initially observed phenotype, which is always a concern when conducting reverse-genetic screens. For this reason, I obtained a second, independent T-DNA insertion mutant for *CAT2* (*cat2-2*) with the aim of characterising the growth of this second line in the presence of alanine (Figure 6.1).

The rosette growth of wild-type is inhibited by approximately 42% in the presence of exogenous alanine, which is consistent with all previous growth experiments (Figure 6.1). The rosette growth of *cat2-2* however, shows no inhibition in the presence of alanine, thus indicating that like *cat2-1*, this line is also insensitive to exogenous alanine. This gives me reasonable confidence that the growth phenotype observed in both these mutant lines is a direct result of the loss-of-function of *CAT2*.

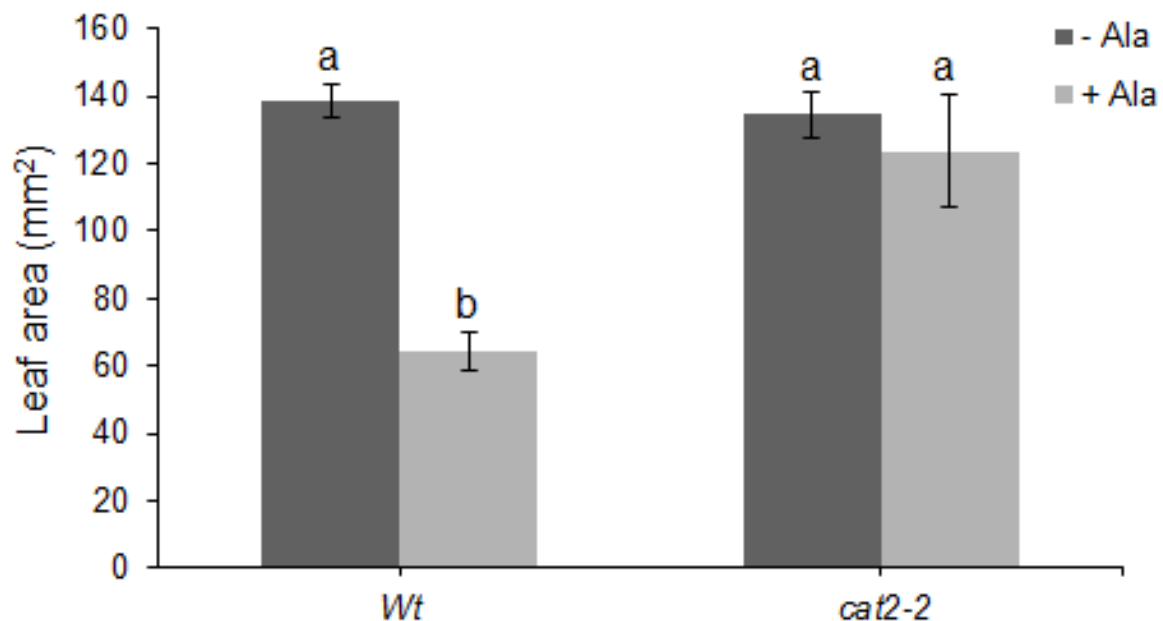


Figure 6.1. The effect of supplying exogenous alanine on rosette growth in the *cat2-2* mutant.

Wild-type and *cat2-2* seeds were germinated on plates containing ½ MS medium. After 11 days, seedlings were transplanted to sand and watered every other day with N-free Hoagland's solution plus 1 mM either NH_4NO_3 or 1 mM NH_4NO_3 + 1 mM alanine. Plants were photographed at 23 days after sowing and leaf area was quantified using Leaf lab. Data are the mean of $n = 12 \pm \text{SEM}$. Significant differences at $p < 0.05$ are indicated by letters (t-test).

To complete the characterisation of *cat2-2*, I wanted to know whether this mutant was also perturbed in nitrogen-status signalling. To determine this, I measured the abundance of the *NRT1.1* transcript in wild-type and the *cat2-2* mutant in response to exogenous alanine (Figure 6.2). Again, I found that the abundance of the *NRT1.1* transcript, relative to the untreated control, was greatly reduced in wild-type. On the contrary, in the *cat2-2* mutant, the abundance of the *NRT1.1* transcript was increased significantly compared to the untreated control, and to wild-type levels (Figure 6.2). This suggests that the perturbation in nitrogen-status signalling can be attributed to the loss-of-function of CAT2 transcript.

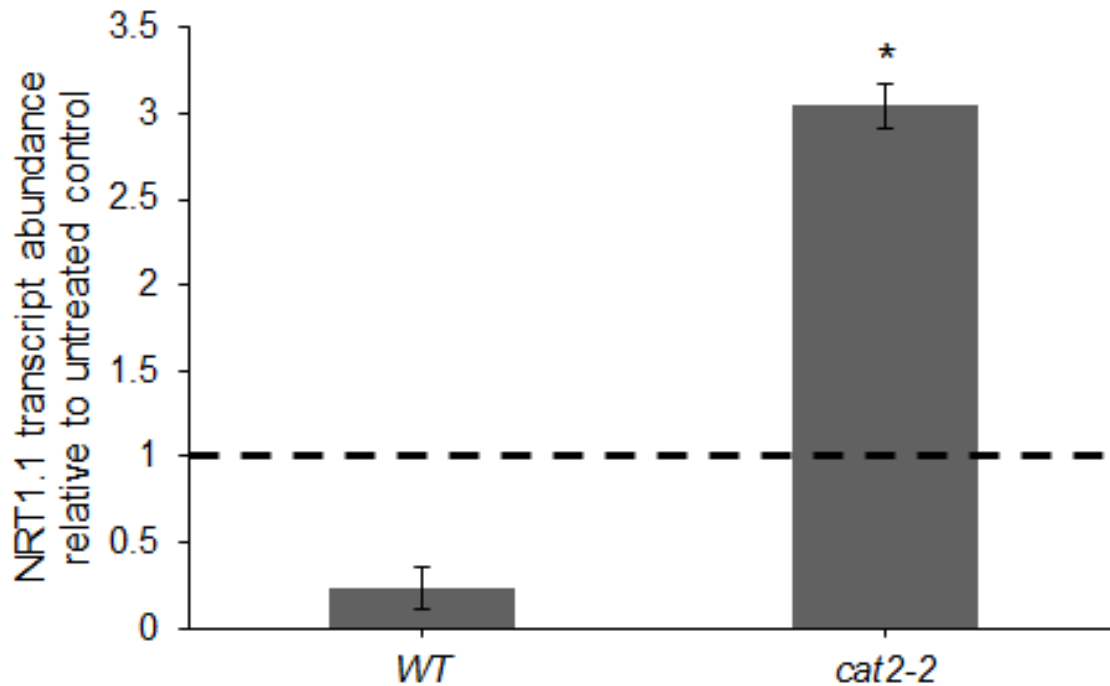


Figure 6.2. The effect of exogenously supplied alanine on NRT1.1 transcript abundance in *cat2-2* and wild-type.

Wild-type and *cat2-2* Arabidopsis seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were starved for 24 hours in flasks containing 0.1 mM CaCl_2 . After 24 hours, 1 mM NH_4NO_3 (untreated control) or 1 mM NH_4NO_3 + 1 mM Ala was added for a further 24 hours. Roots were sampled exactly 24 hours later for RNA extraction. RNA was transcribed to cDNA for qRT-PCR analysis. Transcript abundance was normalised to the housekeeping transcript *TIP41-like* and expressed relative to the untreated control (1 mM NH_4NO_3). Black dashed line represents the untreated control value. Data are the mean of $n = 3 \pm \text{SEM}$. *cat2-2* is significantly different from wild-type at $p < 0.05$ and is indicated by * (t-test).

6.2.2 Investigation into the effect of other key amino acids on the nitrogen signalling response

Many amino acid transporters bind and transport a multitude of amino acids, yet I have only focused on the effect of alanine on the nitrogen signalling response. I therefore wanted to test whether other key amino acids, namely glutamate and glutamine would have the same effect on nitrogen-status signalling as alanine. To accomplish this, I studied the effect of

exogenous glutamate and glutamine on the abundance of the dual affinity nitrate transporter *NRT1.1* transcript in wild-type and *cat2-1* (Figure 6.3).

Similarly to alanine, both exogenous glutamate and glutamine decreased the abundance of the *NRT1.1* transcript in wild-type, in comparison to the untreated control (Figure 6.3 A, B). In the *cat2-1* mutant, however, supplying exogenous glutamine resulted in an increase in the abundance of the *NRT1.1* transcript to levels above that of the untreated control (Figure 6.3 A) and supplying exogenous glutamate resulted in no change in the *NRT1.1* transcript abundance, compared to the untreated control.

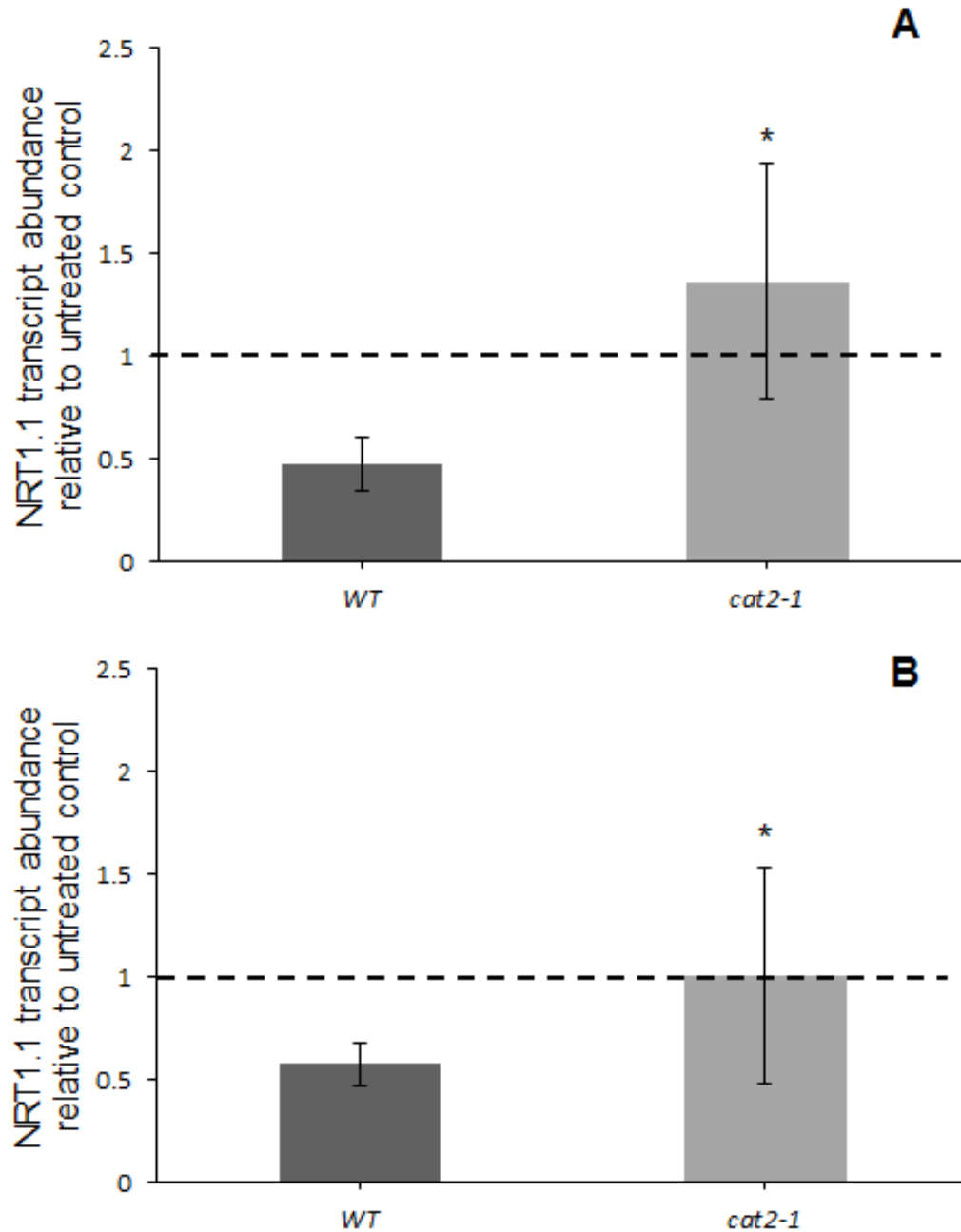


Figure 6.3. The effect of exogenous glutamate and glutamine on NRT1.1 transcript abundance.

Wild-type and *cat2-1* Arabidopsis seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were starved for 24 hours in flasks containing 0.1 mM CaCl_2 . After 24 hours, 1 mM NH_4NO_3 (untreated control) or 1 mM NH_4NO_3 + 1 mM glutamate (A) or 1 mM glutamine (B) was added for a further 24 hours. Roots were sampled exactly 24 hours later for RNA extraction. RNA was transcribed to cDNA for qRT-PCR analysis. Transcript abundance was normalised to the housekeeping transcript *TIP41-like* and expressed relative to the untreated control (1 mM NH_4NO_3). A) Plants fed glutamate; and B) plants fed glutamine. Black dashed line represents the untreated control value. Data are the mean of $n = 3 \pm \text{SEM}$. *cat2-1* is significantly different from wild-type at $p < 0.05$ and is indicated by * (t-test).

6.2.3 Using Microscale Thermophoresis to investigate CAT2 binding substrates

Exogenous glutamate, glutamine and alanine result in nitrogen-status signalling perturbations. To understand further the effect of these amino acids on nitrogen-status signalling, I wanted to test whether they are able to bind to the CAT2 transporter. If the amino acids are able to interact with the transporter, this may indicate that they are directly sensed by the transporter. Therefore, I will use microscale thermophoresis to investigate binding substrates for CAT2.

Creating a temperature gradient in an aqueous solution of biomolecules induces a flow of heat as well as flow of molecules (Talbot et al., 2006). This connection between heat and mass flow is known as the Ludwig-Soret effect of thermophoresis. The effect is sensitive to changes in the size of the molecule, its charge and its conformation, all of which can be altered by ligand binding, making microscale thermophoresis (MST) a sensitive test for molecular interactions (Wienken et al., 2010). Temperature gradients are created using an infrared laser which is coupled to a fluorescence detector. Inside capillary tubes, the fluorescently tagged protein concentration is kept constant, whilst the potential ligand concentration is increased by means of a titration. Before the infrared laser is switched on, the fluorescently-tagged protein will be homogeneously distributed in the capillary, which is recorded as the initial state. Once the infrared laser is switched on, the molecules experience the thermophoretic force in the temperature gradient and typically move out of the heated spot. The depletion of molecules from the heated spot reflects the fraction of bound molecules, which is monitored by the change in fluorescence. The laser is kept on for a few seconds which is usually sufficient for a steady-state to be reached. When the laser is switched off, proteins will diffuse back into initial, homogeneously distributed state. The movement of molecules is monitored by fluorescence detection. Bound proteins will move differently to unbound proteins and this difference will increase proportionally to the amount

of the substrate added, until the protein binding sites are saturated, which makes identifying ligands very simple.

Small quantities of purified protein are required for MST experiments. Therefore, I overexpressed the CAT2 protein fused to GFP and 6xHis in *S.cerevisiae* in large-scale cultures and purified the protein using immobilised metal ion affinity chromatography (IMAC) and size exclusion chromatography (SEC) (Figure 6.4) (see chapter 2.11 for full methods). A sample of total solubilised protein (TS) was taken before beginning IMAC. Samples of the flow-through (FT 1, 2 and 3) from IMAC containing unbound protein were taken to ensure unbound protein had been removed. The CAT2 protein was eluted from the nickel-resin using 250 mM imidazole (E), concentrated by means of a centrifugal concentrator and fractionated by SEC. The peak fraction (P) shows the purified, concentrated CAT2 protein.

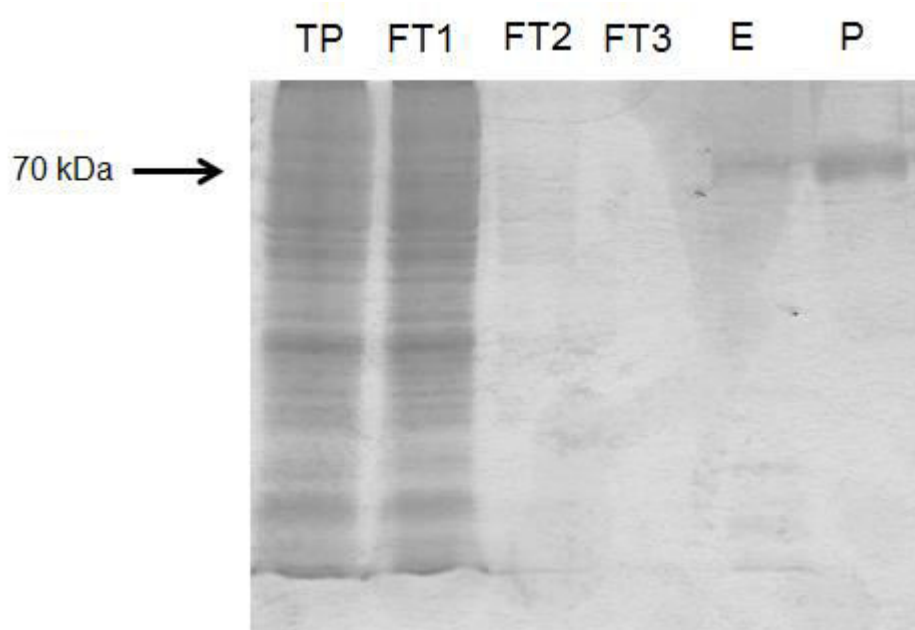


Figure 6.4. Purified CAT2 protein in *S.cerevisiae*.

CAT2-GFP was overexpressed in *S.cerevisiae* and protein was purified using immobilised metal ion affinity chromatography and size exclusion chromatography. Protein samples were collected throughout the purification process and separated by SDS-PAGE. TP = total solubilised protein, FT1 = First flow-through after metal ion affinity chromatography, FT2 = second flow through, FT3 = third throw-through, E = elution from metal ion affinity chromatography and P = peak fraction isolated by size exclusion chromatography.

The peak CAT2 protein isolate was used at a concentration of 0.8 μ M throughout the MST experiment, whereas the amino acid substrates were used at concentrations ranging from 3 – 10 mM. Each amino acid substrate in turn was incubated with the CAT2 protein in a capillary tube and binding was determined based on the how quickly the molecules back diffused into the heat space once the laser was turned off, which was monitored by GFP fluorescence (Figure 6.5).

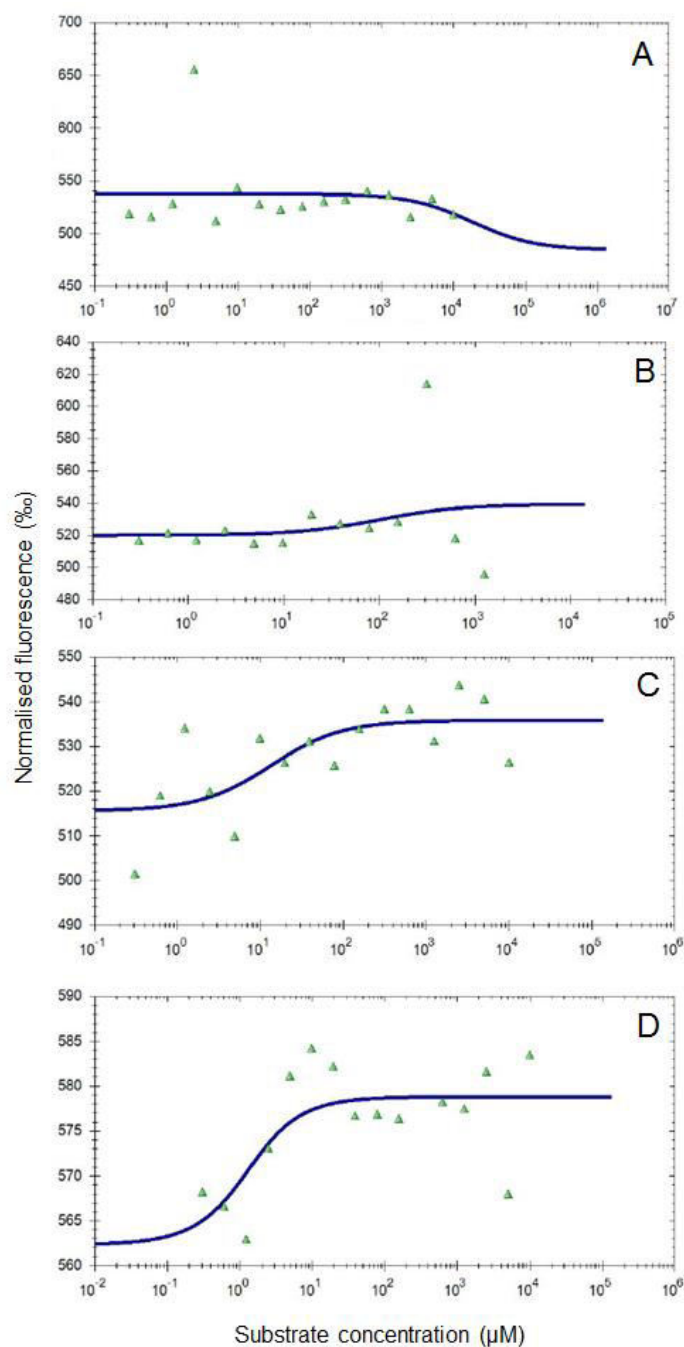


Figure 6.5. Microscale thermophoresis using CAT2 protein to detect substrate binding.

CAT2-GFP protein was incubated with increasing concentrations of alanine (A), glutamate (B) proline (C) and glutamine (D) in capillary tubes. The thermophoretic movement of a fluorescent molecule (CAT2-GFP) changes upon binding to a non-fluorescent ligand (amino acid), resulting in different traces. For analysis, the change in thermophoresis is expressed as the change in normalised fluorescence. Bound protein fluorescence is normalised to unbound protein fluorescence and titration of the non-fluorescent substrate results in a gradual change in thermophoresis, which is plotted as normalised fluorescence (green triangles) to yield a binding curve (blue line), which can be fitted to derive binding constants.

In total, 10 amino acids were tested in the MST experiment. These amino acids were; alanine, asparagine, aspartate, glutamine, glutamate, glycine, histidine, proline, serine and arginine. Out of these 10, only glutamine and proline elicited a fluorescence pattern characteristic of binding to the transporter (Figure 6.5). If a binding-complex is formed, the difference in the level of fluorescence detected when the complex diffuses back into the heat spot after the laser is switched off increases as the substrate concentration increases until the protein binding sites are saturated, as can be seen with proline and glutamine (Figure 6.5 C, D). Conversely, when CAT2 was incubated with alanine or glutamate (Figure 6.5 A, B), fluorescence remains constant across all concentrations of alanine tested, indicating that alanine does not bind to CAT2. This is because bound-protein moves more quickly away from the heat than unbound-protein, and back diffuses more quickly, therefore fluorescence which increases proportional to substrate concentration, after the light is switched off reflects protein-ligand binding.

The change in fluorescence in the focal area can be directly transferred to a binding curve when plotted against substrate concentration and from this the dissociation constant (K_d) can be calculated. From the glutamine-CAT2 binding curve a K_d of $0.889 \mu\text{M} \pm 0.252 \mu\text{M}$ was inferred and from the proline-CAT2 binding curve, a K_d of $12.9 \mu\text{M} \pm 4.59 \mu\text{M}$ was inferred.

6.2.4 Using non-metabolisable analogues of Ala, Glu and Gln to test whether these amino acids need to be metabolised in order to perturb nitrogen-status signalling in *cat2-1*

In this thesis, I have established that exogenous alanine, glutamine and glutamate result in nitrogen-status signalling perturbations. A binding assay revealed that only one of these amino acids, glutamine, appears to bind the CAT2 protein, increasing the likelihood that CAT2 is indirectly involved in perturbing nitrogen-status signalling, rather than directly involved. To further understand the role of the CAT2 transporter in nitrogen-status signalling,

I wanted to test whether the exogenously supplied amino acid required metabolising in order to elicit an effect on nitrogen-status signalling. If they do need to be metabolised, this would be further indicative of the transporter having an indirect on nitrogen-status signalling downstream of the transporter. To answer this question, I fed wild-type and *cat2-1* seedlings non-metabolisable analogues for alanine, glutamine and glutamate and measured the abundance of the *NRT1.1* transcript (Figure 6.6). Methionine sulfoximine (MSX) is glutamate analogue which inhibits glutamine synthetase (Evstigneeva et al., 2003), 6-diazo-5-oxo-L-norleucine (DON) is a glutamine analogue and therefore inhibits the activity of glutamine oxoglutarate aminotransferase (Shapiro et al., 1979), and α -aminoisobutyric acid (AIB) is a structural alanine analogue that will therefore inhibit alanine aminotransferase reactions.

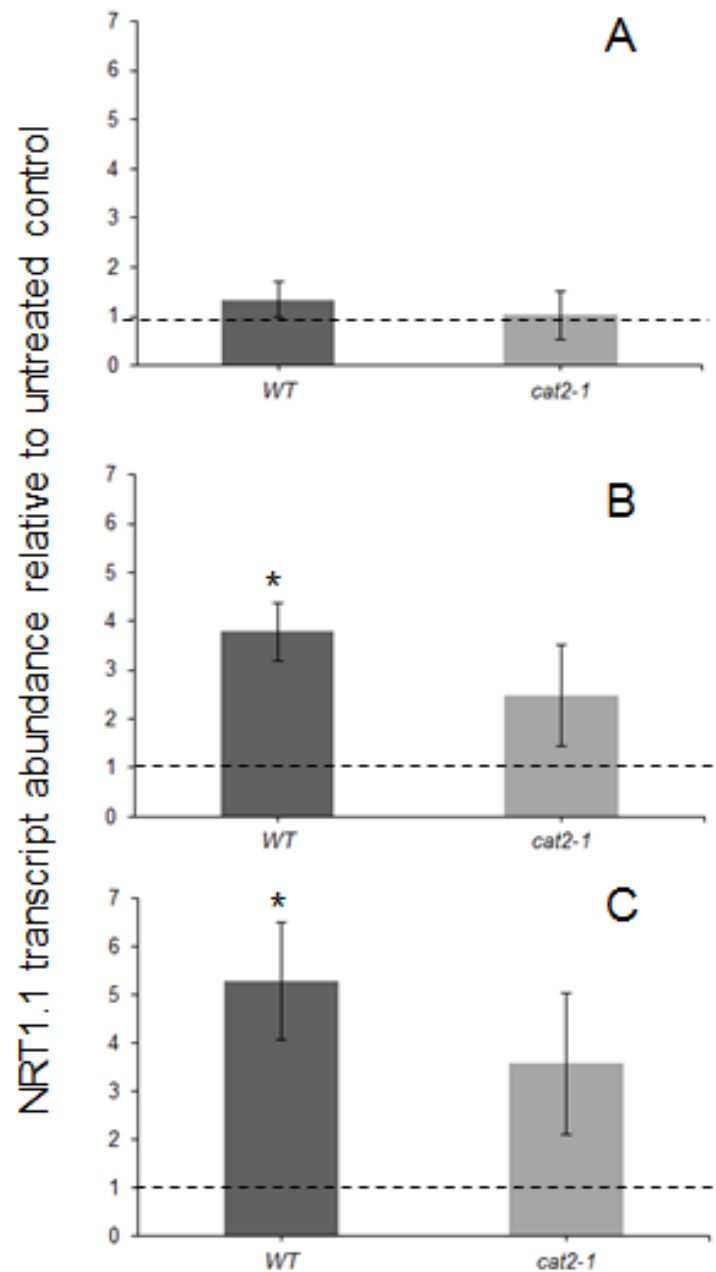


Figure 6.6. The effect of feeding non-metabolisable Glu, Gln and Ala analogues on the abundance of *NRT1.1* transcript in wild-type and *cat2-1*.

Wild-type and *cat2-1* Arabidopsis seeds were germinated on plates containing $\frac{1}{2}$ MS medium. After 11 days, seedlings were starved for 24 hours in flasks containing 0.1 mM CaCl_2 . After 24 hours, 1 mM NH_4NO_3 (untreated control), or 1 mM NH_4NO_3 + 1 mM DON (A); or 1 mM NH_4NO_3 + 1 mM MSX (B); or 1 mM NH_4NO_3 + 1 mM AIB was added for a further 24 hours. Roots were sampled exactly 24 hours later for RNA extraction. RNA was transcribed to cDNA for qRT-PCR analysis. Transcript abundance was normalised to the housekeeping transcript *TIP41-like* and expressed relative to the untreated control. Black dashed line represents the untreated control value. Data are the mean of $n = 3 \pm \text{SEM}$. Values significantly different from the untreated control at $p < 0.05$ are indicated by * (t-test).

No significant differences were observed in *NRT1.1* transcript abundance between wild-type and *cat2-1*, indicating that the response of the mutant to the analogues was the same as wild-type. The response of the *NRT1.1* transcript in wild-type differs from that observed when metabolisable amino acids are used. When the analogues are supplied, no suppression of the *NRT1.1* transcript is observed. In fact, there is a significant induction of *NRT1.1* when MSX is supplied (Figure 6.6 B) and when AIB is fed (Figure 6.6 C).

6.2.5 Investigating protein interactions using Tandem Affinity Purification

Since altering the function of CAT2 in Arabidopsis results in the perturbation of nitrogen-status signalling, it is likely that either CAT2 interacts with components of the nitrogen signalling pathway, or, that the loss-of-function of CAT2 alters the distribution of metabolites within cellular compartments to affect the signalling pathway. To determine whether CAT2 interacts physically with other signalling components, a Tandem Affinity Purification (TAP) tag was fused to the CAT2 protein and overexpressed in the *cat2-1* mutant background (see Chapter 2.8 for detailed methods). C-terminal and N-terminal fusions were created in case the tag interfered with protein function at either one of the termini. This approach will also be used to determine whether the four alanine-insensitive amino acid transporters function as a complex, as if this is true, I should see all four transporter proteins co-purify in this experiment.

The TAP-tag method allows for the purification of complexes containing the TAP-tagged protein. The tag consists of tandem protein A domains that bind IgG-agarose, a tobacco etch virus (TEV) protease site for TEV protease-mediated cleavage of the protein from the IgG-agarose, and a calmodulin-binding protein (CBP) domain for reversible, calcium-dependent binding to calmodulin-agarose (Figure 6.7).

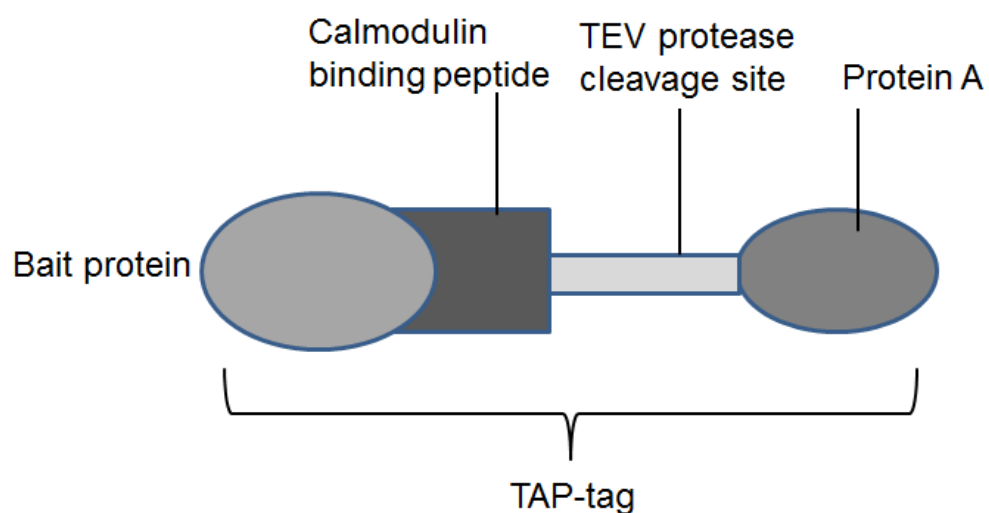


Figure 6.7. Schematic representation of TAP-tag.

The lines most strongly expressing the TAP-tag were selected by immuno-blotting using an antibody (peroxidase anti-peroxidase) which binds to Protein A in the TAP-tag (Figure 6.8). The lines selected for tandem affinity purification were 35S:CTAG-CAT2-1 and 35S:CAT2-NTAG-5 (Figure 6.8) both of which demonstrated a strong protein band at the expected molecular weight of ~90 kDa on the immuno-blots.

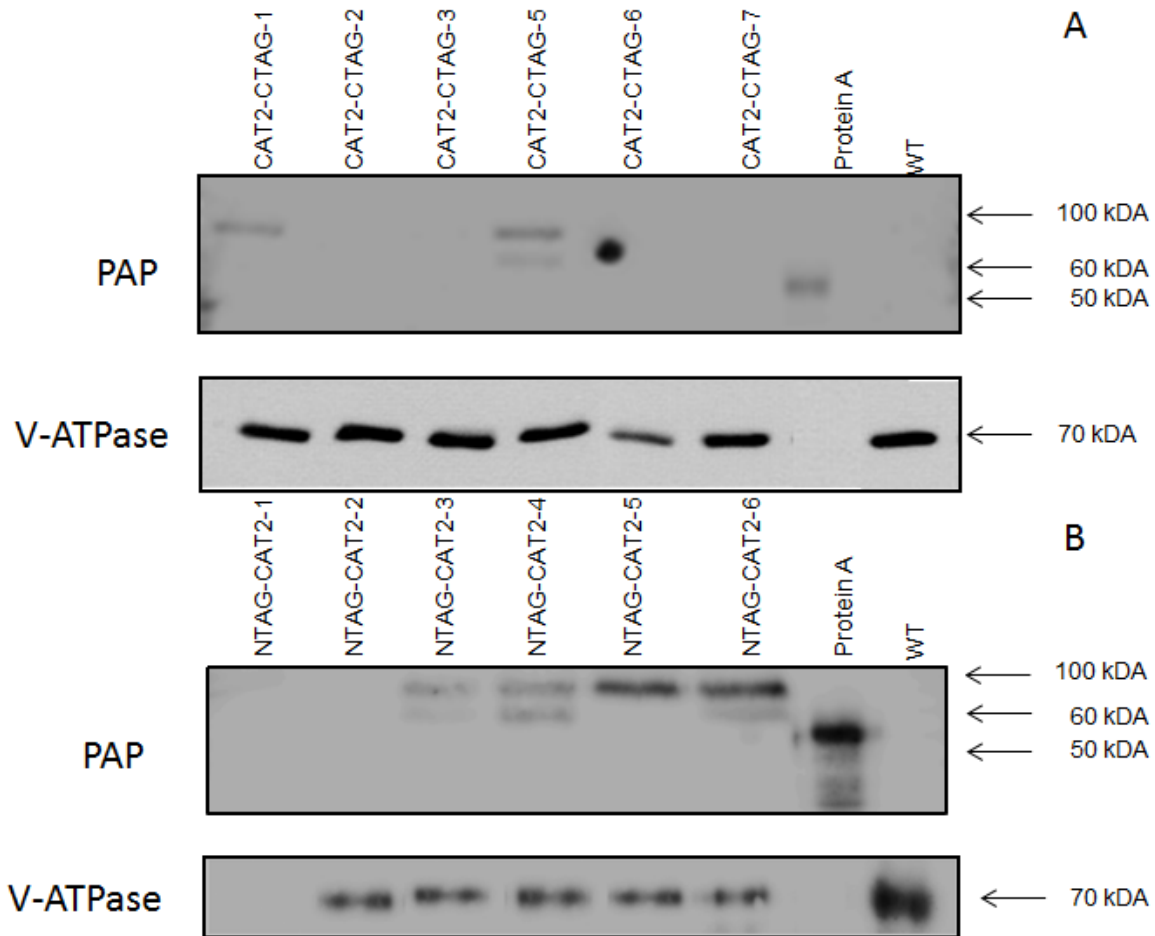


Figure 6.8. Protein A detection in TAP-tag expressing lines using PAP antibody.

Protein extracts from *cat2-1* mutants transformed with 35S:CTAG-CAT2 (A) or 35S:CAT2-NTAG (B) were probed with a peroxidase anti-peroxidase (PAP) antibody, which binds to Protein A in the TAP-tag. V-ATPase was used as a tonoplast loading control. Protein A and wild-type (WT) were included as positive and negative controls, respectively.

Fusing a TAP-tag to a protein of interest can interrupt the function of the protein by causing misfolding or by the tag obscuring binding sites. For this reason it was important to ensure that the CAT2 protein was still functional. To do this I used the established growth assay to test whether overexpressing the CAT2 TAP-tagged proteins in the *cat2-1* mutant background was able to rescue the wild-type growth phenotype (Figure 6.9).

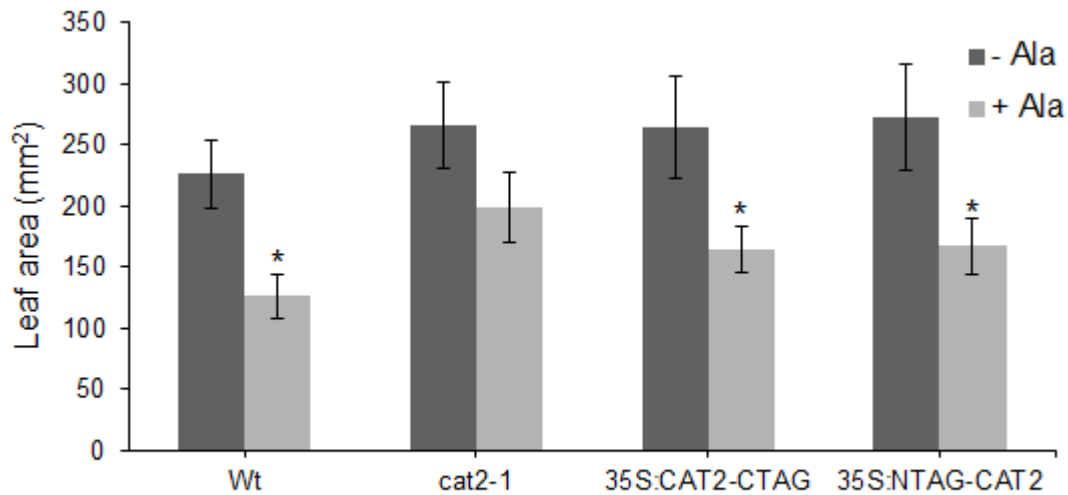


Figure 6.9. Growth assay to determine whether lines overexpressing TAP-tagged CAT2 were able to rescue the wild-type phenotype.

Seeds were sterilised and sown on to plates as previously described. After 11 days, seedlings were transplanted into sand and watered every other day with N-free Hoagland's solution plus 1 mM either NH_4NO_3 or 1 mM NH_4NO_3 + 1 mM alanine. Plants were photographed at 23 days after sowing and leaf area was quantified using Leaf lab. Data are the mean of $n = 12 \pm \text{SEM}$. Significant differences at $p < 0.05$ between "+" and "-" Ala within the same lines are indicated by * (t-test).

The growth assay suggests that the CAT2 protein in the TAP-tagged lines is functional since the vegetative growth in these lines is inhibited by exogenous alanine by approximately 38%, which is similar to the wild-type growth phenotype which is inhibited by approximately 45%, thus perhaps indicating that the wild-type phenotype has been rescued (Figure 6.9). However, the growth inhibition of the complemented lines was not significantly different to that of *cat2-1* which showed an approximate growth inhibition to alanine of 22%. Since the response of nitrate transporter transcripts to exogenous alanine is a much stronger response than growth, arguably a better test of complementation would be to study transcript responses to see whether the wild-type phenotype had been recovered, however there was not time to achieve this.

The TAP-tag experiment was conducted as described in the methods chapter. Briefly, ~60 g of fresh weight of whole plant seedlings were sampled and cross-linked using 1% formaldehyde. Protein was extracted and tonoplast membrane was isolated using cell fractionation. The tonoplast isolate was incubated with IgG-agarose so that binding could occur. TEV protease was used to cleave the CAT2-tagged protein from the IgG beads, which was then incubated with calmodulin-agarose to achieve the second affinity binding step. Protein was eluted from the calmodulin-agarose and separated on an SDS-PAGE gel (Figure 6.10).

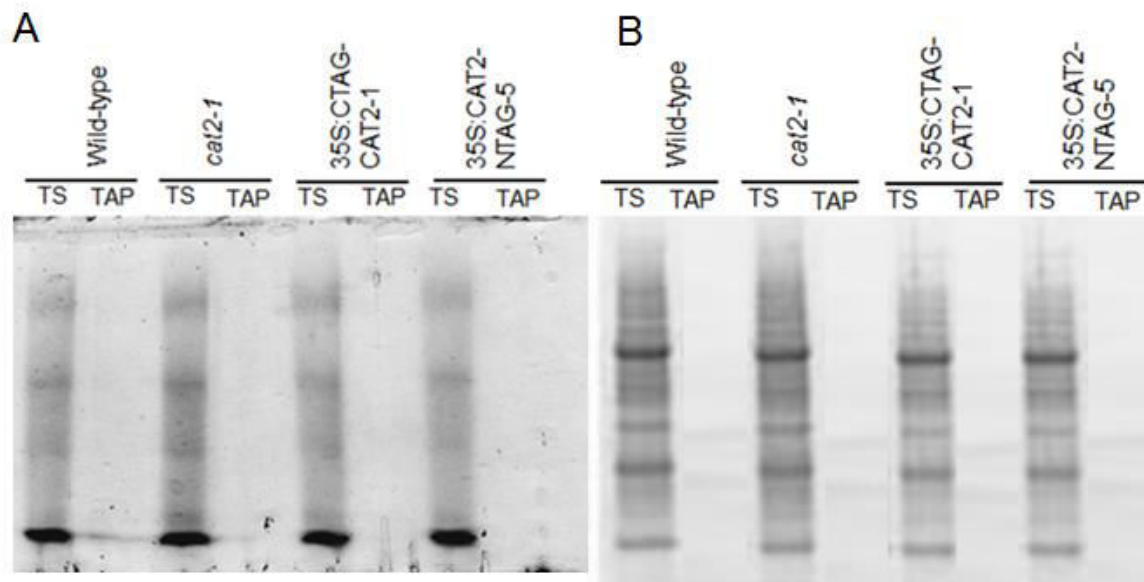


Figure 6.10. Affinity purification using TAP-tagged lines.

Tonoplast protein extracts from TAP-tagged lines, *cat2-1* and wild-type were affinity purified in a two-step process with an IgG antibody which binds to Protein A of the TAG-tag, followed by binding to calmodulin beads (A). Whole protein extracts were affinity purified using just an IgG antibody (B). Protein bound to either IgG or calmodulin beads (TAP = tandem affinity purified protein) was eluted and run on an SDS-PAGE gel, along with total solubilised protein (TS) for each sample.

The TAP-tag experiment was unsuccessful in isolating the CAT2 protein via affinity purification. The results show that the extracted total tonoplast protein was low in abundance and possibly degraded due to the smeary appearance on the gel (Figure 6.10 A). The

experiment was repeated several times in an attempt to optimise the protocol to purify CAT2. In the second attempt, I used whole protein extracts to increase the total protein concentration. I also omitted the second affinity purification step as a large amount of protein can be lost at this stage. Despite both of these changes, I was still unable to purify CAT2. In a last attempt, I repeated the experiment exactly as before, but used Sypro ruby gel stain to detect proteins on the gel as this stain is reported to detect protein concentrations below 1 ng. However, despite the increased sensitivity of the stain, I was still unable to detect purified CAT2.

6.3 Discussion

6.3.1 Knockout of CAT2 results in amino acid insensitivity caused by a range of exogenous amino acids

Amino acid transporters usually transport a broad range of amino acids; therefore it was unlikely that alanine was the only amino acid able to cause perturbations in nitrogen signalling. Indeed, exogenous glutamate and glutamine also lead to the feedback repression of the *NRT1.1* transcript in wild-type plants (Figure 6.3), thus confirming that other exogenous amino acids can result in the same insensitivity phenotype. This was confirmed in *cat2-1* mutants supplied with exogenous glutamate and glutamine.

The nitrogen-status feedback signal generated by feeding exogenous glutamate and glutamine is perturbed in the *cat2-1* mutant (Figure 6.3). The *NRT1.1* transcript was increased significantly compared to wild-type fed exogenous glutamine and glutamate (Figure 6.3). Interestingly, when feeding glutamate, the *NRT1.1* transcript response was similar to that of alanine feeding; the abundance of the transcript was significantly increased in the presence of glutamate compared to the untreated control. Yet, when feeding glutamine, the abundance of the *NRT1.1* transcript abundance was unresponsive to the presence of glutamine in the *cat2-1* mutant plants. This data may indicate that glutamate and alanine elicit a stronger effect on the nitrogen signalling pathway than glutamine, if the level of *NRT1.1* transcript induction correlates with the level signalling response.

Of the three amino acids (alanine, glutamate and glutamine) that cause nitrogen-status feedback signalling perturbations when exogenously supplied to the *cat2-1* mutant, only one, glutamine, is likely to bind CAT2 (Figure 6.5). This suggests that the binding of the amino acid to the transporter is unimportant in generating the feedback signal to inhibit nitrate uptake, and thus hinting towards the transporter having an indirect role in the nitrogen-status signalling pathway.

6.3.2 Amino acids need to be metabolised to perturb nitrogen-status signalling

When dissecting the cause of a signalling perturbation that arises in response to altered metabolite levels, an important task is to ascertain whether the metabolite is sensed directly by the pathway, or whether the manipulated metabolite has an indirect effect on the pathway through a downstream metabolised compound. When dissecting the role of hexokinase in glucose signalling, Jen Sheen showed that metabolism of sugars beyond the phosphorylation of glucose was not required for sugar signalling responses (Jang and Sheen, 1994; Jang et al., 1997). This was based on the findings that the glucose analogues 2-deoxyglucose and mannose, which are not metabolised through glycolysis, can trigger sugar signalling responses (Jang and Sheen, 1994).

Feeding exogenous non-metabolisable glutamine, glutamate and alanine analogues to wild-type and *cat2-1* mutants did not produce the same perturbation in nitrogen signalling as seen when metabolisable amino acids are fed (Figure 6.6). This suggests that the amino acids need to be metabolised in order to affect signalling and therefore implies that the concentration of these amino acids is not sensed directly. Accordingly, this could indicate that another related metabolite, for example, an amino acid, keto acid or other downstream metabolite is sensed in order to generate the signal which affects nitrogen-status signalling. If this is indeed true, this would support the existence of a metabolic signature which is sensed downstream of the amino acid transporters to indirectly alter nitrogen-status signalling.

6.3.3 Identifying proteins that interact with CAT2

Fusing a TAP-tag to the CAT2 protein is a good method to study protein interactions. Transgenic lines overexpressing the tag at both C- and N-termini were successfully generated and the expression of the tag was verified by Western blot analysis (Figure 6.8). Growth analysis of the tagged lines indicated that the CAT2 protein was functional as the

wild-type growth response to exogenous alanine was rescued in these lines (Figure 6.9). I was, however, unsuccessful in purifying the CAT2 protein, along with any interacting proteins during the tandem affinity purification (Figure 6.10). Around 80% of bound protein can be lost between the first and second affinity binding events, therefore to try to increase the concentration of CAT2 to detectable levels, I tried the experiment with just the first, IgG, affinity binding step, but to no avail. It may be that the CAT2-tagged protein was too poorly expressed to be compatible with this experiment. However, further optimisation of the experiment would be necessary before this can be concluded.

6.3.4 Proline and glutamine appear to bind CAT2

The MST binding assay suggests that proline and glutamine are binding substrates for CAT2 (figure 6.5 B, C). Both amino acids produced an increase in fluorescence that is characteristic of a binding interaction. However, this experiment was not replicated therefore to be certain that the result was not an artefact the interaction of these amino acids with CAT2 would need to be studied again. A further limitation of this experiment was that only 10 amino acids were tested. This was due to the amount of available purified CAT2 protein being limiting which could not be resolved in the time-scale of this project. Obviously, to gain a full picture of potential binding substrates, all amino acids would need to be tested. That being said, it is interesting that a mere two out of 10 amino acids elicited a binding response, suggesting that CAT2 may be specific for relatively few transport substrates. Individual amino acid transporters tend to differ in their affinity for substrates, For example, members of the amino acid permease family transport a large spectrum of amino acids (Fischer et al., 1995). In contrast, proline transporters (ProT) as the name suggests have a high affinity for proline (Rentsch et al., 1996). It is clear, therefore, that amino acid transporters range from broad-range to more specific with specialised roles. Since CAT2 transports two out of 10 tested amino acids, it may suggest a more specialised role for this protein.

The ability of a ligand to bind a transporter does not necessarily correlate with the ability to transport it. Therefore, it would be vital to test the functional uptake of CAT2 binding substrates. No transport substrates have been identified for CAT2, but the binding assay suggests that the protein interacts with at least two amino acids. This may suggest that the role of CAT2 has developed from functioning as a transporter to functioning as a sensor, either with little or no transport activity.

The nitrogen signalling perturbation caused by the loss-of-function of CAT2 could be the result of an indirect or direct effect on the pathway. A direct effect suggests that CAT2 interacts with the signalling pathway whereas an indirect effect would suggest that the loss of the transporter affects the subcellular concentration of amino acids, which in turn affects the signalling pathway. Since alanine appears not to bind the transporter (Figure 6.5 A), and because non-metabolisable amino analogues do not elicit a signal, this would indeed suggest that the signalling effect is indirect.

6.4 Summary

The nitrogen signalling perturbations observed in the *cat2* mutants are the result of the loss-of-function of the transporter transcript, and these signalling perturbations can be induced by feeding exogenous alanine, glutamine and glutamate. Analogue studies suggest that these metabolites require metabolising in order to affect the signalling pathway.

A binding assay revealed that proline and glutamine interact with CAT2, however, only 10 amino acids were tested and further functional uptake studies would be required to confirm the transport of these amino acids.

Chapter 7: General Discussion

7.1 Whole plant nitrogen-status regulates nitrate uptake

Plants, being sessile organisms, must regulate the uptake of nitrogen in accordance with its external availability (supply) and internal availability (demand). Plants exposed to nitrate after a period of nitrogen starvation show a strong induction of high affinity nitrate uptake transporter transcripts, whereas these transcripts are down-regulated following a period of sufficient nitrate (Minotti et al., 1969; Jackson et al., 1973; Goyal and Huffaker, 1986; Aslam et al., 1993; Henriksen and Spanswick, 1993; Zhuo et al., 1999). Therefore, it can be concluded that nitrate uptake systems are under the control of both local signalling, triggered by external nitrate and systemic long distance signalling, triggered by whole plant nitrogen-status (Figure 7.1). For example, in *Arabidopsis* split root experiments, nitrogen starvation on one side of the root system leads to an increase in lateral root growth and an upregulation of nitrate uptake on the other side of the root system (Ruffel et al., 2011). This response must be the result of a systemic nitrogen-status signal produced by the shoot in response to the plant being, overall, deficient in nitrogen. This systemic signalling stimulates lateral root growth in the nitrogen-rich compartment and inhibits lateral root growth in the compartment containing no nitrogen in order to maximise nitrate uptake. The origin of this systemic signal is unknown, however, the transduction of the signal is likely to be mediated by cytokinins (Ruffel et al., 2011). Also implicated in the control of root foraging in response to systemic nitrogen-status is the transcription factor TCP20 (Teosinte branched1/cycloidea/proliferating cell factor1-20) (Guan et al., 2014). TCP20 was identified in a yeast-one-hybrid screen as mediating root nitrate foraging through a systemic signalling pathway (Guan et al., 2014). TCP20 was found to bind to more than 100 nitrate responsive promoters in a yeast-one-hybrid screen, whereas insertion *tcp20* mutants were impaired in root foraging in split-root plates. The exact role TCP20 plays in governing root foraging in response to systemic

nitrogen signalling is yet to be discovered, however, the evidence clearly indicates that TCP20 plays a key role in mediating this systemic signalling pathway (Guan et al., 2014).

Thus far, I have mentioned examples of positive regulators of root foraging, i.e. examples which promote root foraging in response to systemic nitrogen-status. However, there are also examples of pathways which suppress root growth in response to nitrogen limitation. For example, small signalling peptides known as C-terminally encoded peptides (CEPs) (Delay et al., 2013) are secreted by roots in response to nitrogen limitation to suppress root growth (Tabata et al., 2014). Under such conditions, CEPs are translocated to the shoot, where they interact with the leucine-rich repeat receptor kinases CEP Receptor1/2 (CEPR1/2) (Tabata et al., 2014). Shoot CEPRs are responsible for CEP-mediated systemic upregulation of nitrate transporters in roots, implying that an unknown secondary signal is transduced from shoot to root.

Mutants impaired in the systemic feedback repression of the high affinity root nitrate transporter NRT2.1 were isolated using a forward genetic screen designed to capture mutants insensitive to high nitrate provision. The mutation was mapped to a region the authors called High Nitrogen Insensitive 9 (HNI9) which encodes Arabidopsis Interacts With SPT6 AtIWIS1, a protein belonging to the RNA polymerase II complex (RNAPII) (Widiez et al., 2011). Therefore suggesting a role for HNI9 in regulating nitrate uptake in response to high nitrate availability.

Clearly the examples listed above indicate the existence of a systemic nitrogen-status signalling pathway. What is unclear, however, is whether there is one global pathway, monitoring total nitrogen levels, or whether there are a series of pathways controlling positive and negative regulation of nitrogen acquisition. It is also possible that different pathways exist for different forms of nitrogen. For example, nitrate, ammonium and amino acids may be sensed individually and may feedback to the same, or different systemic nitrogen-status signalling pathway. As reduced forms of nitrogen, amino acids are ideally placed within the

nitrogen reduction pathway to serve as metabolic signals of nitrogen-status. There is mounting evidence to suggest that amino acids are sensed and this signal transduced as a systemic signal for nitrogen regulation (Behrend and Mateles, 1975; Breteler and A. Arnozis, 1985; Lee et al., 1992; Muller, 1992; Muller et al., 1995; Thornton, 2004; Dluzniewska et al., 2006). The evidence collected in this project suggests that amino acids may be sensed and that this signal may contribute to the systemic signalling of nitrogen-status. However, the mutant phenotype may also arise due to the effects of the mutations on subcellular metabolite abundances. Both hypotheses will be discussed in the key findings section of this chapter.

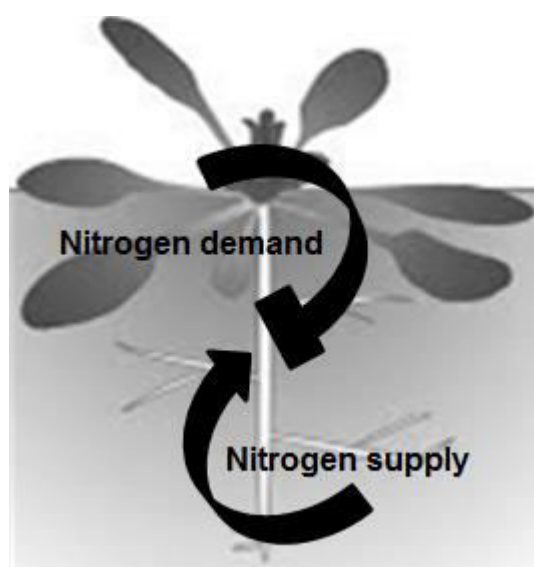


Figure 7.1 Schematic diagram representing systemic nitrogen signalling.

The external availability of nitrate (and possibly ammonium) generates a signal of nitrogen supply. A systemic signal, produced by the plant as a whole, generates a signal of nitrogen demand. As reduced nitrogen forms, an abundance of amino acids may be sensed, which produces a feedback signal to regulate nitrogen uptake (supply) when systemic nitrogen levels are sufficient. When the systemic signalling system detects a low abundance of nitrogen, feedback suppression of nitrogen supply is lifted, promoting nitrogen uptake

7.2 Key findings

Exogenous amino acids inhibit nitrate uptake (Lee et al., 1992; Muller, 1992; Muller et al., 1995) which is consistent with nitrate u3. The work described in chapter 3 also builds on this by showing that exogenous amino acids (alanine, glutamine and glutamate) inhibit vegetative growth as well as transcripts associated with nitrate uptake and assimilation. This supports the existence of a systemic nitrogen demand signal, which, once sufficient nitrogen is detected, can feedback to regulate further nitrogen uptake. The growth response to exogenous alanine was exploited in a reverse-genetic screen in chapter 4 to isolate candidate amino acid transporter T-DNA insertion mutants with perturbed nitrogen-status signalling. The screen identified four mutants with a growth-response that was insensitive to exogenous alanine. Further characterisation of these alanine-insensitive mutants revealed that nitrate uptake was no longer inhibited and *NRT1.1* transcript levels were increased, rather than decreased, in response to exogenous alanine. Furthermore, alanine uptake rates were normal. Taken together, these data may suggest that the systemic nitrogen-status feedback repression of nitrate uptake has been disrupted in alanine-insensitive mutants. Alternatively, the T-DNA insertion mutation in the amino acid transporter mutants could affect metabolite abundances within the plant, either in specific tissues or cell compartments. This would lead to a redistribution of metabolites at a subcellular level, which could potentially be sensed downstream by the nitrogen-status signalling pathway, resulting in the observed phenotype. Thus indicating that the amino acid transporter does not play a direct role in nitrogen-status signalling, but rather the redistribution of metabolites causes the signalling perturbation.

7.3 More amino acids transporters than expected were insensitive to exogenous alanine

Plant growth is variable since it is controlled by a multitude of environmental and internal factors. This variability became apparent when conducting the growth screen in chapter 4. The majority of amino acid transporter T-DNA insertion mutants were observed to be insensitive to exogenous alanine in at least one of the four repetitions of the growth screen. However, only four of the mutants were consistently insensitive to exogenous alanine in all four growth screens. This was unanticipated as I expected an important signalling protein to be unaffected by genetic redundancy, much like the examples of amino acid transporters in animals and plants (Goberdhan et al., 2005; Ho et al., 2009; Rebsamen et al., 2015). The fact that many other amino acid transporter mutants were intermittently insensitive to exogenous amino acids could indicate that the signalling effect is indirect and caused by changes in local amino acid concentrations which are sensed by a second, unknown mechanism. These changes could be highly environment-sensitive and this may explain the inconsistent response of many of the knockout mutants. Amino acid transporter T-DNA insertion mutants will almost certainly alter subcellular metabolite abundances, which may produce differing strengths of a common metabolic signal which is detected elsewhere by a systemic nitrogen demand sensor. It is also possible that amino acids are just one form of nitrogen that feed into the systemic nitrogen signal; perhaps other forms of nitrogen such as nitrate, nitrite and ammonium can also contribute to the nitrogen demand signal. The systemic nitrogen demand signal could be part of a global growth regulatory network which regulates nitrogen-status, carbon-status and energy status to ultimately regulate growth, much like the amino acid transporters interaction with the TOR growth regulatory pathway in animals (Goberdhan et al., 2005; Oegmundsdottir et al., 2012)

7.4 The origin of the nitrogen signalling perturbation

This project outlines some evidence that systemic nitrogen signalling may have been perturbed in alanine-insensitive amino acid transporter mutants. This effect is either the result of a direct effect on the signalling pathway, i.e. the amino acid transporter is itself a component of the pathway; or is the result of an indirect effect of the absence of the amino acid transporter. An indirect effect could arise due to perturbations in local metabolite concentrations, caused by the T-DNA insertion mutation disrupting amino acid transporter function and therefore amino acid transport rates. A disruption in amino acid transport could cause amino acids to be differentially distributed on a sub cellular level, which could affect nitrogen-status sensing downstream of the transporter. The subcellular increase or decrease of some metabolites may well be detected by the nitrogen signalling pathway to produce the observed phenotype. Trehalose-6-phosphate (T6P) is an excellent example of a metabolite that can affect a signalling pathway. T6P can directly inhibit the activity of SnRK1, presumably via a direct interaction with the SnRK1 complex, which results in the promotion of biosynthetic processes (Zhang et al., 2009; Tsai and Gazzarrini, 2014; Nuccio et al., 2015). High sucrose levels result in high T6P levels, an intermediary product in trehalose synthesis, therefore T6P is a sucrose sensor which regulates growth processes based on sucrose availability. T6P provides evidence that metabolite signals do exist and can interact with signalling pathways. Chapter 5 attempted to uncover the origin of a metabolic signal common to alanine-insensitive mutants by using GC-MS, LC-MS to detect metabolite levels, however, no common signature was found among the metabolites that were detected. It remains possible that a metabolite signature within a specific subcellular compartment exists, however, the metabolite quantification methods used in this project could only detect total metabolite levels and could therefore not differentiate between cellular compartments. It is also possible that the build-up of signal is not common in all four alanine-insensitive mutants. For example, deregulation of an allosteric binding site or post-translational modification site, could cause general nitrogen or carbon metabolites to accumulate differently in subcellular compartments, which could perturb the normal signalling of a nutrient-status pathway.

Chapter 6 focused on uncovering proteins that interacted with one of the alanine-insensitive amino acid transporters to determine whether the transporter was directly involved in the systemic nitrogen signalling pathway, or whether the four alanine-insensitive transporters formed a functional complex. However, the overexpressed TAP-tagged protein was too poorly expressed to be detected on a polyacrylamide gel stained with coomassie or sypro red. Thus the position of the alanine-insensitive amino acid transporter within the nitrogen-status signalling pathway remains unconfirmed. The fact that other key amino acids, namely glutamate and glutamine can cause nitrogen-status perturbations, and that they need to be metabolised in order to exert the effect suggest that systemic nitrogen signalling occurs downstream of the transporter. Coupled to this, the fact that the majority of amino acid transporters were observed to have growth insensitive to exogenous alanine in at least one of the four growth-response screens, supports the idea that the signalling perturbation effect occurs as an indirect result of altering localised amino acid levels. One would expect to see a more consistent growth response if the transporter was directly involved in the systemic signalling pathway.

7.5 Implications for engineering NUE in plants

Success in improving NUE by targeting nitrate uptake and assimilation machinery has been limited. For example overexpressing glutamine synthetase (GS) (Kozaki and Takeba, 1996) or nitrate reductase (NR) (Curtis et al., 1999; Djennane et al., 2002a; Djennane et al., 2002b) failed to increase NUE. This lack of success may be explained by the strong regulatory network controlling NUE. Although targeting nitrogen uptake and assimilatory machinery will increase nitrogen supply, systemic nitrogen demand signalling and its feedback regulation of nitrogen supply remains intact. Modulation of this nitrogen demand pathway in combination with overexpression of nitrate uptake and assimilation components would arguably be a better approach to improving plant NUE. The mechanism for nitrogen demand signalling has yet to be discovered. However, the perturbations in this signalling pathway identified in this

thesis may assist in its discovery in the future. Once identified, one approach to improving NUE could be to simultaneously overexpress nitrate uptake transporters in plants with silenced systemic nitrogen demand feedback regulation.

Nitrogen signalling and sugar signalling are intimately linked; indeed increased NUE was inadvertently achieved by overexpressing the sugar transporter STP13 in *Arabidopsis* or the transcription factor DOF1 (Schofield et al., 2009). This link was further highlighted in chapter 5, which observed a greater biomass of alanine-insensitive mutants when grown on media with an additional carbon source (sucrose). These mutants, which may be perturbed in systemic nitrogen-status signalling, are likely to be more nitrogen-use efficient because feedback repression of nitrate uptake is absent. Under normal growth conditions (i.e. under controlled light and no added carbon source), carbon availability is fixed and the growth of the mutants may be limited by this. Increasing carbon supply by adding a carbon source to the media increases the biomass of the mutants, suggesting that NUE will be best achieved by engineering plants with increased flux through nitrogen and carbon metabolism.

7.6 Future work

Deciphering whether alanine-insensitive mutants play a role in systemic nitrogen-status signalling may become clearer if we could uncover whether the transporters have a direct or indirect role in the pathway. Identifying proteins which interact with the transporters would be one way to identify a direct effect. This was attempted in chapter 6 using the TAP-tagged CAT2 protein in a pull-down assay to identify interacting partners; however the assay was unsuccessful and will require further optimisation for it to be successful. It is likely that the tagged CAT2 protein is too poorly expressed which could be overcome by increasing the amount of starting tissue. A more sensitive dye, such as sypro ruby red, may be able to detect smaller quantities of the CAT2 protein than the Coomassie dye. The protein may have been degraded during the extraction process or during the affinity purification. This could be resolved by increasing the concentration of protease inhibitors used throughout the

procedure. This pull-down assay has a strong chance of success after optimisation. The TAP-tag is expressed in transgenic lines, as evidenced by western blot analysis, therefore should be able to be affinity purified. Occasionally, creating a fusion protein can alter the structure or the subcellular localisation of the protein. In which case, the CAT2 protein may not be present in the tonoplast membrane extract, which could be resolved by using whole protein extracts instead.

Alternatively, the alanine-insensitive amino acid transporter mutants may indirectly perturb the nitrogen-status signalling pathway by causing the build-up of a metabolic signal, or simply by causing the redistribution of metabolites, which is sensed downstream of the transporter. I extensively analysed the metabolic profile of mutants and wild-type but no metabolic signature common to the alanine-insensitive mutants could be found. As previously mentioned, the GC-MS and LC-MS methods used in this project could only detect total metabolite levels and could therefore not detect metabolite concentration differences within different cellular compartments. To rule out the possibility of a metabolic signature existing within a particular cellular compartment, organelles would need to be isolated in order to quantify metabolite levels. Cell fractionation could be used to achieve this although this method would be time consuming as well as difficult to ensure that fractions contained only the organelle of interest.

Further investigation into whether the TOR pathway interacts with the systemic nitrogen-status pathway could be achieved by producing double mutants for *tor/cat2-1*, for example. the TOR pathway regulates growth based on the availability of amino acids in animals (Goberdhan et al., 2005; Oegmundsdottir et al., 2012). Therefore, the TOR pathway may interact with the systemic nitrogen-status signalling pathway in order to regulate growth in accordance with amino acid availability.

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Appendix A: Additional information for chapter 3

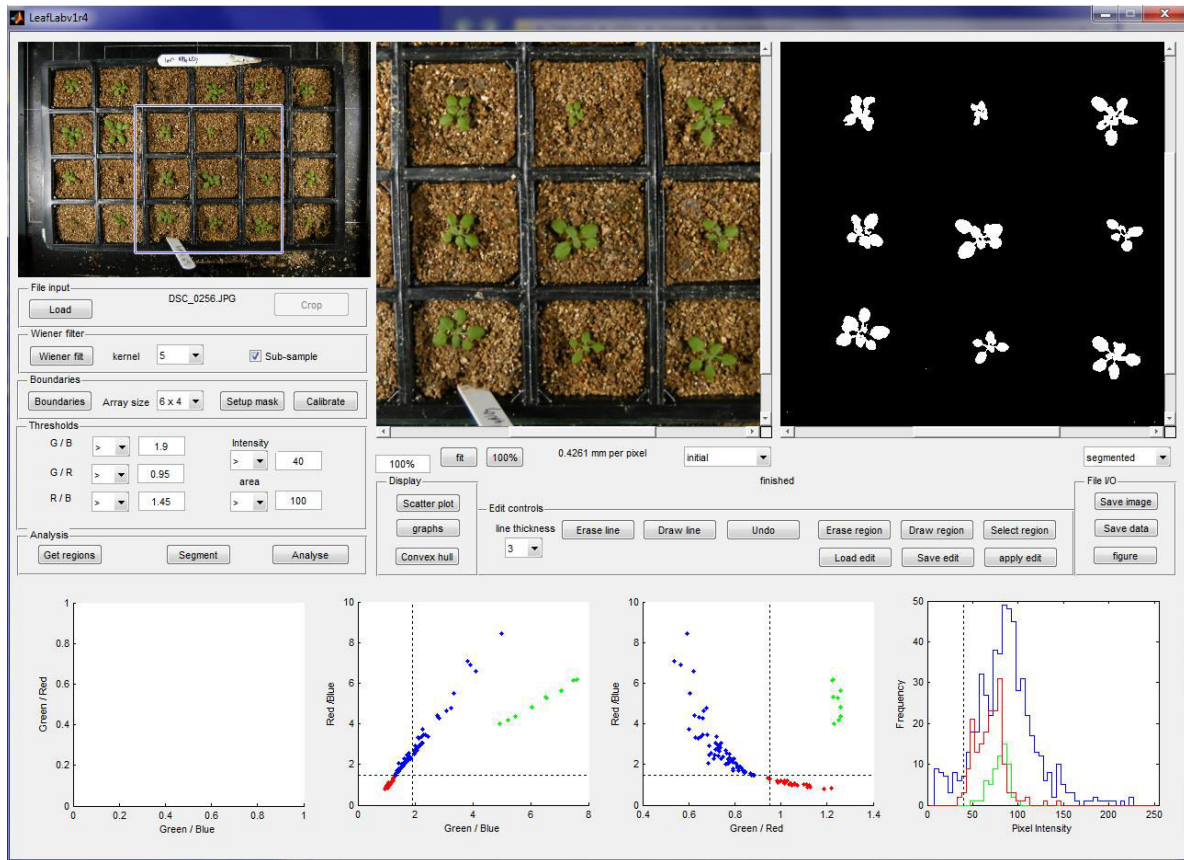


Figure 1: Leaf lab interface

Image shows the user interface of the in-house leaf area analysis software, Leaf lab, which is written in Matlab. The function buttons on the left are used to differentiate between green leaf pixels and soil/tray pixels. Area is calibrated to the width of the tray which produces the output of leaf area in mm^2 . All leaf area analyses in this thesis were conducted using this program.

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
at3g01760-1	NS	Y	NS	N	Larger than WT on 1mM NH ₄ NO ₃	N	Smaller than WT on 4mM NH ₄ NO ₃ + 1mM Ala	N	NS – 2 out of 4 screens	N – 3 out of 4 screens
lht1-1	NS	N	Smaller than WT on 1mM NH ₄ NO ₃	Y	Smaller than WT on 1mM NH ₄ NO ₃	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 4mM Ala	Y	Smaller than WT on 1mM NH ₄ NO ₃ - 2 out of 4 screens	Y – 3 out of 4 screens
lht-like 1-1	NS	N	NS	Y	NS	Y	Smaller than WT on 4mM NH ₄ NO ₃ Larger than WT on 4 mM NH ₄ NO ₃ + 1mM Ala	Y	NS – 3 out of 4 screens	Y – 3 out of 4 screens
at1g25530-1	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala Larger than WT on 1mM NH ₄ NO ₃	Y	NS	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala – 3 out of 4 screens	Y – 4 out of 4 screens

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
at1g47670-1	NS	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	N	Larger than WT under both conditions	Y	NS	Y	Larger than WT on 1mM NH ₄ NO ₃ + 1mM Ala – 2 out of 4 screens	2x Y 2x N
lht7-1	NS	Y	Larger than WT under both conditions	N	NS	Y	Smaller than WT under both conditions	N	NS – 2 out of 4 screens	2x Y 2x N
aap1-1	NS	N	Larger than WT on 1 mM NH ₄ NO ₃	N	NS	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	NS – 2 out of 4 screens	2x Y 2x N
aap2-1	Smaller than WT under both conditions	N	Larger than WT under both conditions	N	NS	N	NS	N	NS – 2 out of 4 screens	N- 4 out of 4 screens
aap5-1	NS	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Larger than WT under both conditions	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala – 3 out of 4 screens	2x Y 2x N

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
at2g40420-1	NS	N	Larger than WT on 1 mM NH ₄ NO ₃	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Smaller than WT under both conditions	Y	Variable result in each screen	2x Y 2x N
at3g30390-1	Larger than WT under both conditions	N	Larger than WT under both conditions	N	Smaller than WT on 1 mM NH ₄ NO ₃ Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	N	Smaller than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala – 3 out of 4 screens	N – 4 out of 4 screens
at2g42005-1	NS	Y	Larger than WT under both conditions	N	Larger than WT under both conditions	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala – 3 out of 4 screens	2x Y 2x N

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
at2g41190-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	N	Larger than WT under both conditions	N	Smaller than WT on 1 mM NH_4NO_3 Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	N	Smaller than WT on 1 mM NH_4NO_3	N	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 3 out of 4 screens	N – 4 out of 4 screens
at5g15240-1	NS	N	Larger than WT on 1 mM NH_4NO_3	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	N – 4 out of 4 screens
at2g39130-1	NS	N	Larger than WT on 1 mM NH_4NO_3	N	NS	N	NS	N	NS – 3 out of 4 screens	N – 4 out of 4 screens
at5g02180-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	NS	Y	Smaller than WT on 1 mM NH_4NO_3	N	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 2 out of 4 screens	Y – 3 out of 4 screens

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
at5g65990-1	NS	Y	Larger than WT under both conditions	N	Larger than WT on 1 mM NH ₄ NO ₃	N	NS	N	Larger than WT on 1mM NH ₄ NO ₃ – 2 out of 4	N – 3 out of 4 screens
bat1-1	NS	N	Larger than WT under both conditions	N	Larger than WT under both conditions	N	NS	N	NS – 2 out of 4 screens Larger than WT under both conditions – 2 out of 4 screens	N – 4 out of 4 screens
cat1-1	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	N	Smaller than WT on 1 mM NH ₄ NO ₃ Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	Y	Smaller than WT on 1 mM NH ₄ NO ₃ + 1mM Ala	N	Larger than WT on 1 mM NH ₄ NO ₃ + 1mM Ala – 2 out of 4 screens	N – 3 out of 4 screens

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
cat2-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT under both conditions	Y	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 3 out of 4 screens	Y- 4 out of 4 screens
cat3-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT under both conditions	N	NS	N	Smaller than WT on 1 mM NH_4NO_3	N	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 2 out 4 screens	N – 3 out of 4 screens
cat4-1	Smaller than WT on 1 mM NH_4NO_3	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	N	Smaller than WT under both conditions	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 2 out of 4 screens	Y – 3 out of 4 screens
cat6-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT under both conditions	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	NS	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 2 out of 4 screens	Y – 4 out of 4 screens

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
cat8-1	NS	Y	Larger than WT under both conditions	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 3 out of 4 screens	Y – 4 out of 4 screens
cat9-1	NS	Y	Larger than WT under both conditions	Y	Larger than WT under both conditions	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	N	Larger than WT under both conditions – 2 out of 3 screens	2x Y 2x N
ptr2-1	NS	N	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	NS	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	N	NS – 2 out of 4 screens	N – 3 out of 4 screens
ptr4-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Smaller than WT under both conditions	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala – 2 out of 4 screens	Y – 3 out of 4 screens

Appendix B: Qualitative summary of all screen results from chapter 4

Gene ID	Screen 1		Screen 2		Screen 3		Screen 4		Most reproducible result	
	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?	Rosette size	Insensitive to Ala?
ptr6-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT on 1 mM NH_4NO_3	N	Smaller than WT on 1 mM NH_4NO_3 + 1mM Ala	N	NS	N	Variable in each screen	N – 3 out of 4 screens
at5g40670-1	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	Larger than WT under both conditions	N	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y	NS	Y	Larger than WT on 1 mM NH_4NO_3 + 1mM Ala	Y – 3 out of 4 screens

Appendix C: Metabolite concentration table to supplement chapter 5

Table 3: Quantification of metabolites in alanine fed alanine-insensitive mutants, wild-type and *bat1-1* using GC-MS and Tagfinder analysis.

Analyte	Normalised arbitrary abundance mg ⁻¹ FW					Wt
	<i>bat1-1</i>	<i>cat2-1</i>	<i>cat6-1</i>	<i>cat8-1</i>	<i>lht9-1</i>	
Aconitic acid. cis- (3TMS)	0.0008 ± 0.0001	0.0010 ± 0.0002	0.0007 ± 0.0001	0.0007 ± 0.0000	0.0011 ± 0.0001	0.0007 ± 0.0002
Alanine 3-cyano- (2TMS)	0.0058 ± 0.0008	0.0112 ± 0.0020	0.0053 ± 0.0013	0.0070 ± 0.0015	0.0110 ± 0.0014	0.0045 ± 0.0011
Alanine N-acetyl- DL- (1TMS)	0.0013 ± 0.0002	0.0017 ± 0.0002	0.0014 ± 0.0003	0.0013 ± 0.0002	0.0020 ± 0.0002	0.0011 ± 0.0004
Alanine. beta- (3TMS)	0.0034 ± 0.0007	0.0078 ± 0.0021	0.0030 ± 0.0002	0.0043 ± 0.0009	0.0036 ± 0.0005	0.0049 ± 0.0012
alpha-D-Glucopyranosyl- (1.6)-D-glucitol (9TMS)	0.0016 ± 0.0002	0.0016 ± 0.0003	0.0015 ± 0.0003	0.0011 ± 0.0002	0.0011 ± 0.0001	0.0013 ± 0.0004
Arabinose (1MEOX) (4TMS) BP	0.0432 ± 0.0072	0.0363 ± 0.0066	0.0494 ± 0.0050	0.0383 ± 0.0122	0.0560 ± 0.0088	0.0347 ± 0.0130
Arginine [-NH3] (2TMS)	0.1325 ± 0.0383	0.1864 ± 0.0547	0.1131 ± 0.0381	0.1127 ± 0.0288	0.0720 ± 0.0104	0.1144 ± 0.0238
Arginine [-NH3] (3TMS)	0.3891 ± 0.0887	0.5951 ± 0.1051	0.4894 ± 0.0554	0.4376 ± 0.0488	0.4271 ± 0.0241	0.5932 ± 0.1572
Ascorbic acid (4TMS)	0.0007 ± 0.0002	0.0016 ± 0.0005	0.0015 ± 0.0002	0.0012 ± 0.0002	0.0012 ± 0.0001	0.0007 ± 0.0001
Asparagine (3TMS)	0.4037 ± 0.0527	0.3172 ± 0.0546	0.3663 ± 0.0455	0.1843 ± 0.0101	0.1959 ± 0.0278	0.4410 ± 0.1087
Asparagine (4TMS) BP1	0.0043 ± 0.0014	0.0027 ± 0.0005	0.0028 ± 0.0004	0.0018 ± 0.0002	0.0022 ± 0.0003	0.0030 ± 0.0006
Aspartic acid (3TMS)	0.0005 ± 0.0002	0.0003 ± 0.0001	0.0003 ± 0.0001	0.0001 ± 0.0000	0.0003 ± 0.0001	0.0004 ± 0.0001
Aspartic acid. N-(3-indolylacetyl)- (4TMS)	0.0008 ± 0.0001	0.0009 ± 0.0002	0.0007 ± 0.0001	0.0006 ± 0.0002	0.0006 ± 0.0001	0.0009 ± 0.0003
Cellobiose. D- (1MEOX) (8TMS) MP	0.0040 ± 0.0007	0.0038 ± 0.0005	0.0042 ± 0.0014	0.0023 ± 0.0007	0.0044 ± 0.0005	0.0045 ± 0.0017
Cysteine. N-acetyl- (3TMS)	0.0665 ± 0.0086	0.0526 ± 0.0093	0.0602 ± 0.0073	0.0308 ± 0.0018	0.0332 ± 0.0048	0.0719 ± 0.0179
Dehydroascorbic acid (2MEOX) BP	0.0282 ± 0.0058	0.0380 ± 0.0050	0.0510 ± 0.0127	0.0325 ± 0.0052	0.0447 ± 0.0056	0.0296 ± 0.0082
Dotriacontane. n-	0.3676 ± 0.0622	0.5664 ± 0.1391	0.3079 ± 0.0367	0.3566 ± 0.1001	0.2846 ± 0.0383	0.4663 ± 0.1089
Galactose. 2-deoxy- (1MEOX) (4TMS) MP	0.0072 ± 0.0008	0.0064 ± 0.0011	0.0065 ± 0.0005	0.0042 ± 0.0006	0.0064 ± 0.0004	0.0062 ± 0.0019
Galactose-6-phosphate (1MEOX) (6TMS) BP	0.0010 ± 0.0003	0.0014 ± 0.0002	0.0008 ± 0.0004	0.0006 ± 0.0001	0.0007 ± 0.0001	0.0012 ± 0.0005
Gentiobiose (1MEOX) (8TMS) MP	0.1201 ± 0.0219	0.0963 ± 0.0211	0.1188 ± 0.0137	0.1768 ± 0.0904	0.1133 ± 0.0181	0.0808 ± 0.0269
Gluconic acid. 2-amino-2-deoxy- (6TMS)	0.0003 ± 0.0000	0.0003 ± 0.0001	0.0003 ± 0.0000	0.0003 ± 0.0001	0.0004 ± 0.0001	0.0003 ± 0.0001
Gluconic acid. 2-amino-2-deoxy (7TMS)	0.0017 ± 0.0005	0.0012 ± 0.0003	0.0010 ± 0.0001	0.0021 ± 0.0007	0.0010 ± 0.0002	0.0019 ± 0.0008
Gluconic acid. 2-amino-2-deoxy- (7TMS)	0.0009 ± 0.0002	0.0009 ± 0.0001	0.0010 ± 0.0001	0.0010 ± 0.0001	0.0009 ± 0.0001	0.0011 ± 0.0003
Glucose-6-phosphate. 2-amino-2-deoxy- (6TMS)	0.2413 ± 0.1049	0.3695 ± 0.0353	0.4431 ± 0.0682	0.3318 ± 0.0379	0.3672 ± 0.0481	0.4838 ± 0.1485
Glutamic acid (2TMS)	0.0105 ± 0.0032	0.0091 ± 0.0024	0.0098 ± 0.0029	0.0089 ± 0.0018	0.0160 ± 0.0021	0.0053 ± 0.0019
Glutamic acid (3TMS)	1.0183 ± 0.1605	1.0359 ± 0.1439	1.0572 ± 0.0921	0.7160 ± 0.0610	0.9588 ± 0.0632	1.1172 ± 0.3521
Glutamine. DL- (4TMS)	0.0047 ± 0.0006	0.0035 ± 0.0004	0.0048 ± 0.0009	0.0026 ± 0.0003	0.0048 ± 0.0005	0.0026 ± 0.0004

Analyte	Normalised arbitrary abundance ⁻¹ mg FW ⁻¹					Wt
	<i>bat1-1</i>	<i>cat2-1</i>	<i>cat6-1</i>	<i>cat8-1</i>	<i>lht9-1</i>	
Glyceric acid (3TMS)	0.0012 ± 0.0001	0.0010 ± 0.0001	0.0011 ± 0.0002	0.0008 ± 0.0001	0.0011 ± 0.0001	0.0009 ± 0.0003
Glycerol (3TMS)	0.4533 ± 0.0594	0.6597 ± 0.1024	0.5326 ± 0.0824	0.4317 ± 0.0536	0.6179 ± 0.0310	0.4616 ± 0.1399
Heptadecanoic acid (1TMS)	0.7958 ± 0.1356	1.5120 ± 0.3645	0.8188 ± 0.0946	1.0044 ± 0.3036	0.7941 ± 0.1269	1.1236 ± 0.2742
Homoserine beta. DL (3TMS)	0.5681 ± 0.0947	0.6905 ± 0.0806	0.6470 ± 0.0932	0.4563 ± 0.0430	0.6783 ± 0.0429	0.6073 ± 0.1820
Hydroxyurea (3TMS)	0.1439 ± 0.0209	0.2084 ± 0.0356	0.1443 ± 0.0270	0.1541 ± 0.0262	0.2342 ± 0.0169	0.1240 ± 0.0431
Isomaltose (1MEOX) (8TMS) MP	0.0193 ± 0.0015	0.0154 ± 0.0029	0.0209 ± 0.0022	0.0112 ± 0.0011	0.0199 ± 0.0015	0.0180 ± 0.0047
Lactose. alpha- (1MEOX) (8TMS) MP	0.0023 ± 0.0003	0.0040 ± 0.0009	0.0030 ± 0.0006	0.0026 ± 0.0006	0.0020 ± 0.0003	0.0028 ± 0.0008
Leucine (2TMS)	0.0038 ± 0.0005	0.0047 ± 0.0008	0.0044 ± 0.0007	0.0029 ± 0.0002	0.0050 ± 0.0003	0.0037 ± 0.0011
Lyxose (1MEOX) (4TMS) BP	0.0432 ± 0.0072	0.0363 ± 0.0066	0.0494 ± 0.0050	0.0383 ± 0.0122	0.0560 ± 0.0088	0.0347 ± 0.0130
Malonic acid (3TMS)	0.0011 ± 0.0007	0.0005 ± 0.0001	0.0005 ± 0.0003	0.0003 ± 0.0001	0.0003 ± 0.0001	0.0004 ± 0.0001
Maltose (1MEOX) (8TMS) BP	0.0030 ± 0.0004	0.0048 ± 0.0011	0.0041 ± 0.0005	0.0033 ± 0.0005	0.0032 ± 0.0004	0.0044 ± 0.0013
Mannitol (6TMS)	0.0458 ± 0.0078	0.0371 ± 0.0067	0.0362 ± 0.0016	0.0211 ± 0.0029	0.0397 ± 0.0035	0.0350 ± 0.0099
Melibiose (1MEOX) (8TMS) MP	0.0166 ± 0.0018	0.0158 ± 0.0026	0.0207 ± 0.0027	0.0115 ± 0.0012	0.0175 ± 0.0015	0.0176 ± 0.0048
Mevalonic acid-1.5- lactone (1TMS)	0.0080 ± 0.0012	0.0148 ± 0.0025	0.0068 ± 0.0016	0.0098 ± 0.0022	0.0155 ± 0.0019	0.0048 ± 0.0013
Nonacosane. n-	0.3335 ± 0.0554	0.5511 ± 0.1363	0.2957 ± 0.0378	0.3594 ± 0.1020	0.2815 ± 0.0407	0.4258 ± 0.0994
Nonadecanoic acid (1TMS)	0.0027 ± 0.0004	0.0048 ± 0.0012	0.0029 ± 0.0003	0.0035 ± 0.0010	0.0029 ± 0.0004	0.0038 ± 0.0010
Nonanoic acid (1TMS)	0.0023 ± 0.0001	0.0036 ± 0.0007	0.0022 ± 0.0004	0.0028 ± 0.0005	0.0037 ± 0.0004	0.0017 ± 0.0006
Norleucine (3TMS)	5.0424 ± 0.4872	6.5157 ± 1.1553	5.6697 ± 1.0762	4.7904 ± 0.6034	5.1493 ± 0.3205	6.1825 ± 1.7516
Octanoic acid. n- (1TMS)	0.4533 ± 0.0594	0.6597 ± 0.1024	0.5326 ± 0.0824	0.4317 ± 0.0536	0.6179 ± 0.0310	0.4616 ± 0.1399
Ornithine (3TMS)	0.0715 ± 0.0252	0.1019 ± 0.0173	0.1330 ± 0.0418	0.0742 ± 0.0117	0.1115 ± 0.0167	0.0760 ± 0.0256
Ornithine-1.5 lactam (2TMS)	0.0884 ± 0.0094	0.1583 ± 0.0201	0.1769 ± 0.0294	0.1555 ± 0.0066	0.1996 ± 0.0188	0.0855 ± 0.0262
Oxaloacetate (1MEOX) (2TMS) MP	0.0234 ± 0.0041	0.0400 ± 0.0092	0.0223 ± 0.0029	0.0266 ± 0.0072	0.0220 ± 0.0028	0.0305 ± 0.0073
Palatinose (1MEOX) (8TMS)	0.1201 ± 0.0219	0.0963 ± 0.0211	0.1188 ± 0.0137	0.1768 ± 0.0904	0.1133 ± 0.0181	0.0808 ± 0.0269
Pentadecane. n-	0.0275 ± 0.0049	0.0485 ± 0.0118	0.0258 ± 0.0031	0.0315 ± 0.0100	0.0252 ± 0.0035	0.0375 ± 0.0091
Pentatriacontane. n-	0.1480 ± 0.0233	0.2098 ± 0.0514	0.1114 ± 0.0119	0.1200 ± 0.0332	0.0950 ± 0.0123	0.1834 ± 0.0418
Phenylalanine (2TMS)	0.0515 ± 0.0041	0.0579 ± 0.0101	0.0439 ± 0.0045	0.0405 ± 0.0088	0.0544 ± 0.0041	0.0380 ± 0.0123
Proline (2TMS)	0.0003 ± 0.0000	0.0005 ± 0.0001	0.0003 ± 0.0000	0.0004 ± 0.0001	0.0003 ± 0.0000	0.0004 ± 0.0001
Pyruvic acid (2TMS)	0.0844 ± 0.0143	0.1973 ± 0.1053	0.1346 ± 0.0601	0.0426 ± 0.0173	0.0475 ± 0.0098	0.1246 ± 0.0291

Analyte	Normalised arbitrary abundance ⁻¹ mg FW ⁻¹					Wt
	<i>bat1-1</i>	<i>cat2-1</i>	<i>cat6-1</i>	<i>cat8-1</i>	<i>lht9-1</i>	
Ribose (1MEOX)	0.0432 ±	0.0363 ±	0.0494 ±	0.0383 ±	0.0560 ±	0.0347 ±
(4TMS) BP	0.0072	0.0066	0.0050	0.0122	0.0088	0.0130
Siloxane	0.0004 ±	0.0011 ±	0.0003 ±	0.0005 ±	0.0004 ±	0.0004 ±
	0.0001	0.0003	0.0001	0.0001	0.0001	0.0001
similar to Fructose	0.0038 ±	0.0054 ±	0.0048 ±	0.0037 ±	0.0068 ±	0.0038 ±
Derivate	0.0003	0.0011	0.0006	0.0006	0.0005	0.0009
Succinic acid (2TMS)	0.0018 ±	0.0009 ±	0.0018 ±	0.0006 ±	0.0006 ±	0.0017 ±
	0.0002	0.0003	0.0004	0.0001	0.0000	0.0004
Sucrose (8TMS)	0.0010 ±	0.0007 ±	0.0006 ±	0.0003 ±	0.0004 ±	0.0016 ±
	0.0002	0.0001	0.0002	0.0001	0.0001	0.0006
Threose (1MEOX)	0.0071 ±	0.0121 ±	0.0113 ±	0.0104 ±	0.0126 ±	0.0092 ±
(3TMS) MP	0.0005	0.0013	0.0018	0.0007	0.0010	0.0023
Trehalose. alpha.alpha'-	5.3077 ±	4.7298 ±	4.6060 ±	3.8876 ±	5.2728 ±	4.8073 ±
. D- (8TMS)	0.7385	0.5411	0.3451	0.6458	0.7155	1.3742
Tricosane. n-	0.3884 ±	0.6367 ±	0.3491 ±	0.4165 ±	0.3357 ±	0.4967 ±
	0.0619	0.1544	0.0412	0.1168	0.0452	0.1178
Tyrosine (2TMS)	0.0044 ±	0.0025 ±	0.0032 ±	0.0033 ±	0.0046 ±	0.0022 ±
	0.0011	0.0002	0.0005	0.0005	0.0006	0.0008
Valine (2TMS)	0.0630 ±	0.0414 ±	0.0481 ±	0.0308 ±	0.0618 ±	0.0354 ±
	0.0112	0.0095	0.0139	0.0077	0.0063	0.0125
Xylose (1MEOX)	0.0432 ±	0.0363 ±	0.0494 ±	0.0383 ±	0.0560 ±	0.0347 ±
(4TMS) BP	0.0072	0.0066	0.0050	0.0122	0.0088	0.0130

TMS = trimethylsilyl derivatives

MEOX = O-methylhydroxylamine hydrochloride derivatives