Using a chemical genetics approach to dissect the nitrogen signalling pathway in Arabidopsis

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Abstract

Nitrate is an important nutrient and signalling molecule to plants. As it is taken up and assimilated, reduced forms of N accumulate and the expression of many genes associated with nitrate assimilation are repressed. Little is known about the mechanisms involved in this N repression. This project, for the first time, adopts a chemical genetics approach to investigate the feedback regulatory pathway that links the plant's N status to expression of the *NRT2.1* nitrate transporter gene. A novel chemical screening platform was developed that was designed to be used in conjunction with Arabidopsis lines expressing luciferase reporter genes in roots. This semi-hydroponic platform allows roots to be exposed to a variety of nutrient treatments in a 96-well plate format suitable for chemical genetic screens. This was combined with a newly developed 'ice capture' method that provided a rapid and efficient way to harvest root material for the luciferase assay. Using this screening platform in conjunction with a nitrate-inducible luciferase reporter line, *pNRT2.1::LUC*, three chemical libraries, containing 7420 bioactive molecules were screened in duplicate for compounds that antagonise N repression of luminescence. The screen identified a plant-derived alkaloid, camptothecin, that enhanced *pNRT2.1::LUC* expression under N-repressive conditions. The positive effect of camptothecin on expression of the endogenous *NRT2.1* gene was confirmed using real-time PCR and shown to extend to other N-repressed genes of the nitrate assimilatory pathway. Camptothecin is known to target topoisomerase I, an enzyme that is increasingly being linked to a role in chromatin re-modelling, in addition to its more familiar roles in DNA replication and repair. The possible epigenetic role of topoisomerase I in repression of *NRT2.1* and other genes of the nitrate assimilatory pathway is discussed. It was also observed that an arginine treatment strongly stimulated *pNRT2.1::LUC* in the luciferase assay, in a nitrate-dependent manner. Since this effect was not observed at the mRNA level, it is hypothesised that arginine was acting on *pNRT2.1* expression at a post-transcriptional level.

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Academic Thesis: Declaration of Authorship

I, Lucas Gent declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original work.

Title:

Using a chemical genetics approach to dissect the nitrogen signalling pathway in Arabidopsis

Signed: …………………………………………………………………………

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Abbreviations

ACC	Aminocyclopropanecarboxylic acid
Ala	Alanine
ANOVA	Analysis of Variance
AOA	Aminooxyacetate
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
At	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
AZA	Azaserine
BBSRC	Biotechnology and Biological Sciences Research Council UK
bp	base pairs
BSA	Bovine serum albumen
bZIP	Basic leucine zipper
cDNA	Complimentary DNA
CEP	C - terminally encoded peptide
CEPD1	CEP Downstream 1
CEPD2	CEP Downstream 2
CEPR1	CEP Receptor 1
ChIP	Chromatin immunoprecipitation
Col	Columbia

CPT	Camptothecin
Cys	Cysteine
D	Day
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
EtOH	Ethanol
Fig.	Figure
GFP	Green fluorescent protein
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
GS-GOGAT	glutamine synthetase-glutamate synthase
GUS	β-glucuronidase
H	Hour
H^+	Hydrogen ion
H3K27m3	histone 3 lysine 27 trimethylation
HATS	High-affinity transport system
His	Histidine
HNI	High Nitrogen Insensitive
HY5	Elongated Hypocotyl 5

Ile	Isoleucine
IWSI	Interact With SPT6
KNO_3	Potassium nitrate
LSD	Least significant difference
LATCA	Library of AcTive Compounds on Arabidopsis
LATS	Low-affinity transport system
Leu	Leucine
Log	Logarithmic
LOPAC	Library of Pharmacologically Active Compounds
LUC	Firefly luciferase gene
Lys	Lysine
Met	Methionine
Min	Minutes
mRNA	Messenger RNA
MSX	L-Methionine sulfoximine
N	Nitrogen
N-ind	Nitrate induced
NiR	Nitrite reductase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_3^-	Nitrate
NPF	NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER
NR	Nitrate reductase

NRP	Nitrate-inducible promoter
NRQ	Normalised Relative Quantification
NRT	Nitrate transporter
P2Y	purinergic G protein-coupled
PcG	Polycomb Group Protein
PCR	Polymerase chain reaction
PDMS	Plant Design Management System
pH	Potential of Hydrogen
Phe	Phenylalanine
Pro	Proline
RLU	Relative light units
RPM	Revolutions per minute
RNA	Ribonucleic acid
SE	Standard error
Ser	Serine
TAE	Tris Acetate EDTA
T-DNA	Transfer DNA
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
UK	United Kingdom
USA	United States of America
V	Volts

| Val | Valine |
| YFP | Yellow fluorescent protein |

Chapter 1. Literature review

1.1 Introduction

In the year 2000 global starvation rates were at an all-time high, with 900 million people around the world undernourished (FAO, 2017). By 2050 the human population is predicted to reach nearly 10 billion people (FAO, 2009). This increased population will require more food and space, increasing pressure on land already used for agriculture and limiting its geographical expansion (FAO, 2017). This presents a rapidly approaching food security challenge which must be tackled urgently.

This food security challenge has been recognised for some time now. So far humanity has taken a plethora of different approaches to try and reduce the number of people currently starving. Advancements in agricultural output have helped to tackle this issue, contributing to reduction of the 900 million starving people in 2000 down to 777 million in 2015 (FAO, 2017). However, since then the number of starving people has increased, with 815 million people being recorded as undernourished in 2016 (FAO, 2017). This number is currently rising with each passing year and if the human race is to be sustained an effective solution to food production must be found and implemented.

With each coming year farmers are applying larger amounts of nitrogenous-rich fertiliser to their soils to support major crops, such as wheat and rice, in an effort to meet the ever growing demands (Lu and Tian, 2017). To put this into perspective, the application of nitrogen (N) fertiliser has increased roughly 10-fold between 1950 and 2008 (Stuart et al., 2013). At present in 2018, N fertilizer application is calculated to be above 200 million tonnes per year, with a predicted annual growth rate of roughly 1.8% in the future (FAO, 2018). The volume of N fertiliser applied around the world varies greatly, with countries such as China, the United States and India accounting for over 50% of all fertiliser used globally (Lu and Tian, 2017) A major factor for these countries is their large population sizes which exert high demands on agricultural land to meet their food requirments. The supply from farmers is considerably lower than the growing demands for food which has led to the excessive use of N fertiliser in the field in order to close the gap (Lu and Tian, 2017). This over-application is being employed by farmers specifically in an attempt to alleviate any N-limiting

conditions in the field. Of all N fertiliser currently applied it is believed that only 50% is recovered by agricultural crops, while the rest is either left in the soil or transferred away from the site of application (Stuart et al., 2013, Stuart et al., 2014).

Fertiliser can leave the agricultural site of application via air, ground water and surface water pathways (Stuart et al., 2014, Robertson and Groffman, 2015). In places such as the United Kingdom, which have a wet climate for large portions of the year most of the N fertiliser is predominantly leeched from the soil via the water pathways. The predominant form of N in agricultural fertilisers is nitrate, an essential macronutrient for plants which also acts as a powerful signalling molecule, rapidly reprogramming many genes in the N signalling pathway of plants (Li et al., 2017, Masclaux-Daubresse et al., 2010). Nitrate as a charged ion is readily mobile in the soil, with leaching accounting for up to 70% of nitrate loss from the targeted field site (Hodge et al., 2000). Consequently, this provides a transient window in which the crops can absorb and utilise this N source. One solution to this issue is improve the N use-efficiency of the crops, enabling them to maximise use of the available nitrate at a given time (Hirel et al., 2007, Li et al., 2017). This excessive use of fertiliser in combination with leeching is proven to have a great number of environmental and ecological issues (e.g. causing soil acidification and degradation, producing algae blooms in water sources) (Lu and Tian, 2017). While an increased application of N to the fields is being used as a solution for farmers a greater presence of N is not necessarily the best solution. Research has shown that excessive fertiliser use can also have a negative impact on crop yield. This is the case as the uptake, transport and assimilation of nitrate within plants is tightly regulated by their N signalling pathway, which is both induced and repressed by a variety of conditions and internal regulatory factors (Li et al., 2017, Masclaux-Daubresse et al., 2010).

This thesis sets out to explore and learn more about the N signalling pathway in plants. By further dissection of N signalling in plants and its regulatory components the aim is to offer a novel insight into its regulatory mechanisms, helping to inform the world on how plants respond to nitrate in the soil. This would provide an opportunity to tailor N fertiliser application to crops in the field providing an alternative to excessive methods of application that are currently used, which are insufficient for both now and the future. This will help alleviate the number of

undernourished people currently in the world and reduce the number to come as the human race continues to expand. Additionally, alternatives to the excessive application of N fertiliser will have beneficial knock-on effects for both the environment (e.g. reduce the large carbon foot-print associated with fertiliser production) and its ecosystems (e.g. favourable conditions in agronomic soils, less pollution of aquatic ecosystems) (Lu and Tian, 2017).

In this chapter a literature review is presented, providing an in-depth overview of the current understanding of N signalling. It also establishes the foundations of the research reported in this thesis, justifying the approach taken to learn more about the N signalling pathway in plants.

1.2 The Rothamsted experiments

Rothamsted Research (UK) is a science research institute in the UK. It is home to one of the oldest and arguably one of the most important long term experiments in agronomic sciences. This experiment began in 1843 when two scientists, John Lawes and Joseph Gilbert planted winter wheat (*Triticum aestivum L.*) and turnips (*Beta vulgaris L.*) at the Broadbalk and Barnfield locations, respectively (Johnston and Poulton, 2018). The initial goal was to investigate the crops' response to different forms of N, along with other inorganic elements (including N, P, K, Na and Mg) in a systematic manner. Crop tissue material and soil samples were collected to determine the response to different N treatments consisting of organic manure and inorganic fertiliser. The two main outcomes of early experimentation were: 1. Plant responses to available N were dependent on available on availability of phosphorus (P). Observations showed that under P deficiency conditions crops were unable to respond to changes in N treatments. 2. Plants responded differently to inorganic fertilisers compared to the application of organic N. It was observed that a small application of inorganic fertiliser, containing a suitable concentration of N would result in the same yields as when large amounts of organic manure were applied (up to 35 tonnes per hectare) (Johnston and Poulton, 2018). Of these two discoveries, the latter observation was monitored over the years into the 1970s where it was noted that despite an organic N accumulation in the soil of up to two and a half times more than when the first observations were made (this increase was due to an annual application

of organic manure), inorganic fertilisers were still able to produce similar yields at lower application volumes. The conclusion derived from this work at the time was that inorganic forms of N were more important than the supposed 'non-essential' organic N (Johnston and Poulton, 2018). However, in recent years the continuation of the experiment has contradicted this initial finding, with organic N producing higher yields, believed to be the case as organic N improves the soil pH and soil structure in favour of crops establishing good root systems that allow them to access more of the available nutrients and water available, which in turn resulted in higher yields (Johnston and Poulton, 2018).

Microplots within the Broadbalk fields have been isolated for the purposes of studying N cycling. These studies, using an N isotope (N^{15}) reported that of all the fertiliser applied, approximately 50% was taken up by the crops. Of the remaining 50%, half remained in the soil and the other half was believed to either have been leeched out or been converted into other forms by soil bacteria (Powlson et al., 1986; Johnston and Poulton, 2018). Another study confirmed that the loss of N^{15} was predominately due to it being converted by denitrifying bacteria (Jenkinson and Parry, 1989), which was backed up by a model analysing the turnover of N through the soil microbial biomass (Shen et al., 1989). This highlights the role of bacteria in the conversion of forms of N in the soil and more importantly draws attention to the variety of different forms and its availability within soils of both a natural and agronomic context.

1.3 The processes of the N cycle

In the environment N is present in various forms, being converted from one to another by a variety of microorganisms, a process which is encompassed under the concept of the N cycle (Fig. 1.1). The N cycle can be separated into four main processes: ammonification; nitrification; denitrification; annamox (Stein and Klotz, 2016; Berg et al., 2002).
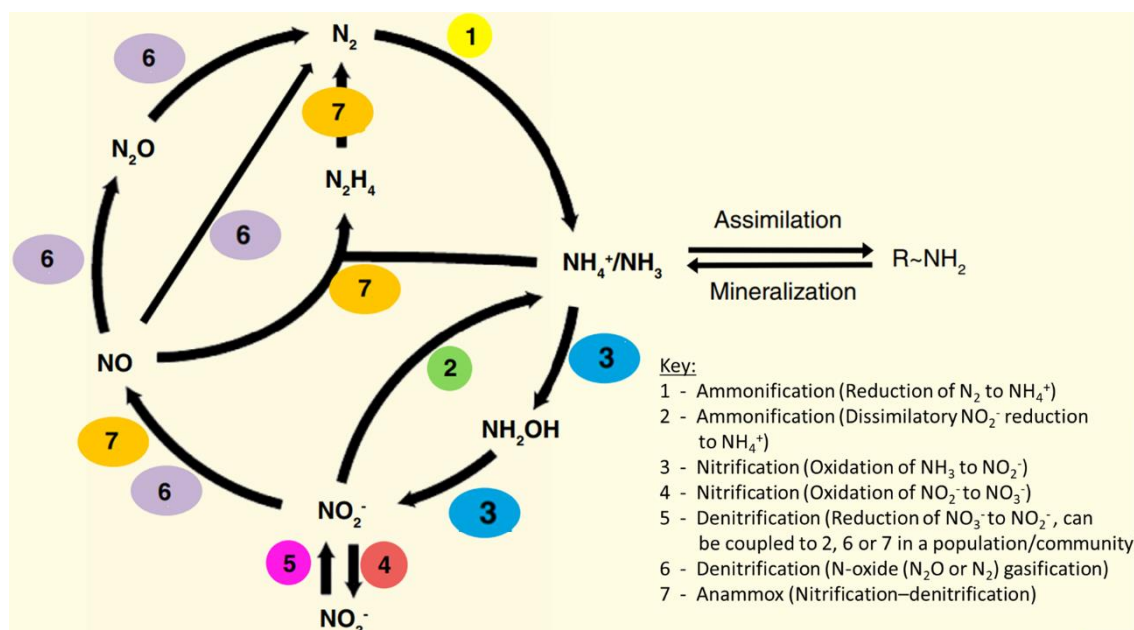
Figure 1.1 Processes involved in the N cycle showing the biological activity of N-cycling microorganisms to convert N into the various forms found in the environment. Figure adapted from Stein and Klotz (2016).

Ammonification is a process performed in bacteria and archea which contain a nitrogenase complex made up of molybdenum–iron protein dinitrogenase and vanadium or an iron protein dinitrogenase reductase in the presence of oxygen. This process allows these organisms to obtained N from the atmosphere through a process known as N fixation in which ammonium is produced (Stein and Klotz, 2016; Berg et al., 2002). These bacteria and archea can then assimilate this ammonium into biomass through respiration. Alternatively, this ammonium can respired by other microbes in an aerobic or anaerobic depend manner. Some bacteria and fungi are responsible for another type of ammonium production, where nitrate is taken up and reduced to ammonium through either aerobic or anaerobic assimilation (Stein and Klotz, 2016).

Nitrification is performed by three main groups of microorganisms. The first group concerns microorganisms which oxidise ammonia to produce nitrite. These organisms are known as ammonia oxidisers and include chemolithotrophs,

heterotrophic and methanotrophic microorganisms (Stein and Klotz, 2016; Berg et al., 2002). The second group concerns microorganisms which oxidise nitrite to produce nitrate. These organisms are known as nitrite oxidisers and consist of only chemolithotrophs, which utilise nitrite as their lone source of energy for cellular growth. The third group concerns microorganisms that oxidise ammonia to nitrate. These microorganisms are known as complete ammonia oxidisers and only include chemolithotrophs which use ammonia as their lone source of energy to support cellular growth. Unlike the chemolithotrophs, heterotrophic and methanotrophic microorganisms do not utilise energy from the conversion of N to sustain cellular growth and development (Stein and Klotz, 2016).

Denitrification is performed by classical or canonical denitrifiers under anaerobic conditions. These microorganisms use respiration to convert nitrite (N_2O^-) to nitric oxide (NO) and nitrous oxide (N_2O) and in turn into nitrogen gas (N_2). However, not all microorganisms in this group, such as the ammonia-oxidising chemolithotrophs encode the necessary enzymes required to make the full conversion from nitrite to N_2, resulting in the release of NO and N_2O into the environment (Stein and Klotz, 2016).

Annamox is a term used to describe anaerobic ammonium oxiders. This is a process which is only performed by *Brocadiaceae* bacteria as they contain annamoxosomes. The presence of this specialised organelle allows them to convert NO_2^- and NH_4^+, first into an NO and hydrazine intermediate and then into N_2. During this conversion there is no production of N_2O (Stein and Klotz, 2016).

There is also another instance in which bacteria play a role in N cycle, however this process occurs symbiotically with plants. Legumes, such as soybeans and alfalfa contain root nodules containing *Rhizobium* bacteria. In this relationship the bacteria converts N_2 into mineral forms of N which are utilised by the plant, while the bacteria benefit from the energy obtained in the conversion to support growth and development (Mokhele et al., 2012; Berg et al., 2002).

This description of the N cycle serves as an opportunity to reflect on the multiple forms of N that exist within soil. Bacteria nitrification accounts for the reduction of a significant amount of N fertiliser applied by farmers, presenting the

crops with a soil environment containing multiple different forms of mineral N. The response of plants to different forms of N is varied, as will be discussed later on.

1.4 Nitrate uptake, transport and regulation

N is an essential macronutrient for plants, it is found at the most fundamental level of biology, in DNA, making up nitrogenous bases which form nucleotides, all the way up to proteins which are used as cell materials and plant tissues (Mokhele et al., 2012). N in both natural and agronomic contexts is usually the main limiting factor for growth and development in plants (Geisseler et al., 2010; Vitousek and Howarth, 1991). In an agronomic context the predominant form of N available to plants is nitrate (Li et al., 2017, Masclaux-Daubresse et al., 2010). In the following section the physiology of nitrate uptake and transport around the plant will be described. As the important role of *NRT2.1* in relation to nitrate becomes clear the literature will focus on the N-dependent regulation of both this gene and its transporter's function.

1.4.1 Physiology of nitrate uptake

As sessile organisms, higher plants have adapted a root system architecture with a high degree of plasticity in response to nutrient availability to overcome their immobility (Smith and De Smet, 2012). Early physiological studies reported that net influx of nitrate into the roots was determined by three uptake systems. The first is a constitutive low-affinity transport system (cLATS), where the presence of nitrate stimulates the transporter function, but only to a small degree. The second is a nitrate-inducible high-affinity transport system (iHATS), where the presence of nitrate strongly stimulates the transporter function. The third is a constitutive high-affinity transport system (cHATS), where the presence of nitrate stimulates the transporter function, but only to a small degree. (Aslam et al., 1992, Glass and Siddiqi, 1995, Forde and Clarkson, 1999).

1.4.1.1 Physiology of the cLATS The LATS play a major role in nitrate uptake at concentrations ≥ 1 mM and its activity in barley has been reported to be non-saturable up to 100 mM in nitrate media depletion experiments (Glass et al., 1990, Siddiqi et al., 1990). Electrophysiological evidence from barley reported that the mechanism of LAT uptake was similar to the mechanism described for the iHATS, with a charge-coupling stoichiometry of 2 H^+ ions taken up for every

NO$_3^-$ molecule imported (Glass et al., 1992). The same research also reported that the LATS were expressed in the absence of nitrate, and were only stimulated by 80% upon nitrate supply (Glass et al., 1992, Forde and Clarkson, 1999). Experiments in barley also showed that, like the iHATS the LATS are subject to repression by reduced forms of N, such as glutamine (Gln), a major end product of N assimilation (Siddiqi et al., 1990).

1.4.1.2 Physiology of the iHATS The iHATS were first observed in barley nitrate depletion experiments, using ^{13}NO$_3^-$ and ^{15}NO$_3^-$ (Clarkson, 1996, Lee and Drew, 1986), showing that nitrate is taken up at low concentrations for the first few hours of exposure and their activity was inducible up to 50-fold (Glass et al., 1992). Similarly this was also shown in Brassicaceae and other catch crop species reporting that the larger the shoot : root ratio the more rapid the uptake rate of nitrate over this time (Laine et al., 1993). After the first few hours of exposure nitrate pooling in the cytosol increased efflux of nitrate from the roots, therefore this method of studying nitrate influx of the iHATS was only suitable in the early stages of study (Forde and Clarkson, 1999). Electrophysiology experiments in barley roots and *Lemna gibba* (duckweed) fronds reported that the fresh supply of nitrate to N starved plants resulted in a transient depolarisation of the membrane potential across the plasma membrane (Glass et al., 1992, Ullrich and Novacky, 1981). In Arabidopsis, similar findings were observed, with increased uptake activity when the roots were in an acidic environment. This observation helped unveil a charge-coupling stoichiometry of two H$^+$ ions for every NO$_3^-$ molecule imported by the iHATS in the root cells (Meharg and Blatt, 1995). This H$^+$ ion importation with nitrate into the cells was hypothesised to lower the pH of the cytoplasm. To investigate this, the cytosolic pH of *Limnobium stoloniferum* root cells were monitored with intracellular electrodes (Ullrich and Novacky, 1990). Instead of acidifying, the pH of the cell cytosol was alkalinised. However, this increase in pH was thought to be the result of nitrate reductase activity (NR) (Forde and Clarkson, 1999). To gain a further insight the pH close to the root surface was measured, it was hypothesised that as the roots took up nitrate, and consequently H$^+$ ions, the media immediately surrounding the roots would alkalise. However unexpectedly they did not observe alkalisation, this was thought to arise from the pH measurement underestimating the removal of H$^+$

ions from the media by 50% (Mistrik and Ullrich, 1996). This understanding of nitrate uptake and its charge-coupling stoichiometry was consolidated in Arabidopsis by using all the described measurement techniques in unison, leading to the production of a kinetic cycle model of the transport system in roots (Meharg and Blatt, 1995). In this model the charge-coupling stoichiometry was confirmed, with 2 H^+ taken up for every NO_3^- molecule imported and the rate of nitrate uptake was shown to be limited by the rate at which negatively charged sites on the newly unloaded nitrate transporter could transition from the inside to the outside of the cell, to access fresh nitrate in the external media (Meharg and Blatt, 1995, Forde and Clarkson, 1999). As well as being nitrate inducible iHATS activity has also been shown to be repressed by reduced amino acids, end products of N assimilation (Lee and Drew, 1986).

1.4.1.3 Physiology of the cHATS In barley the application of nitrate allowed the functioning of iHATS to be observed, but not the cHATs. The cHATS were initially only observed in the absence of nitrate, possessing a higher affinity for nitrate than the iHATS, but with a minimal nitrate uptake capacity (Aslam et al., 1992, Siddiqi et al., 1990, Lee and Drew, 1986, Forde and Clarkson, 1999). In barley and spruce the cHATS activity was shown to be nitrate inducible, however this induction was approximately 3-fold, a small proportion of the stimulated activity in the iHATS and the cLATS (Aslam et al., 1992). It was hypothesized that as the cHATS are expressed in the absence of N application their passive uptake of nitrate could be simply to absorb a significant concentration of nitrate to induce the iHATS and other N-signalling genes in the N assimilation pathway (Forde and Clarkson, 1999).

1.4.2 The NRT 1 and NRT2 families of nitrate transporters

In Arabidopsis there are nine low-affinity nitrate transporter (*NRT1*) and seven high affinity nitrate transporter (*NRT2*) gene homologues that encode proteins involved in nitrate uptake and transport around the plant (Okamoto et al., 2003). NRT1.1/CHL1, now designated NPF6.3 as a member of the NPF (NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER) family is a known dual-affinity nitrate transporter, which also has an important role as a nitrate sensor (Leran et

al., 2014). AtNRT1.1 is located on the plasma membrane of root tip cells (Fig.1.2), with the gene expressed in the epidermis of root tips and in more mature parts of the root (Huang et al., 1996). AtNRT1.2 is constitutively expressed in root cells on the plasma membrane and contributes to nitrate uptake as part of the LATS system (Huang et al., 1996). Once nitrate is taken up into the roots, the AtNRT1.5 transporter, located on the plasma membrane of root pericycle cells near to xylem vessels is responsible for delivering nitrate from the root to the shoot (Lin et al., 2008). Other functions of NRT1 transporters include transporting nitrate into leaf petioles (AtNRT1.4), developing tissues, such as the embryo (AtNRT1.6) (Chiu et al., 2004, Almagro et al., 2008, Masclaux-Daubresse et al., 2010), nitrate transport into parenchymal tissues (AtNRT1.3) (Tong et al., 2016), nitrate remobilisation around the plant (AtNRT1.7) (Fan et al., 2009) and the downward transport of nitrate to the roots (AtNRT1.8 and AtNRT1.9) (Wang and Tsay, 2011; Masclaux-Daubresse et al., 2010).

Regarding the NRT2 family, AtNRT2.1 is of particular importance as it makes up the nitrate inducible element of the HATS. *AtNRT2.1* encoding the NRT2.1 protein which is situated on the plasma membrane of roots cells (Fig. 1.2) is responsible for approximately 75% of total root nitrate uptake (Li et al., 2007, Nazoa et al., 2003, Gansel et al., 2001). AtNRT2.2 is another important inducible nitrate transporter involved in the uptake of exogenous nitrate into the roots, paired with NRT2.1 they contribute up to 80% of total nitrate uptake into the roots. NRT2.4 is located on plasma membranes in the root epidermis and near to shoot phloem contributing to nitrate uptake and transport around the plant, but only in the very high-affinity range under N starvation conditions (Kiba et al., 2012). Other functions of NRT2 transporters include transporting nitrate into the vacuole of cells and into seed (AtNRT2.7) (Chopin et al., 2007) and transporting nitrate into the phloem (AtNRT2.5) (Lezhneva et al., 2014). The genes encoding AtNRT2.3 and AtNRT2.6 share a 91% nucleotide identity, yet the specific functioning within plants is yet to be determined (Dechorgnat et al., 2012).

When nitrate is taken up by the roots it is reduced in the N assimilation pathway. First, inorganic nitrate is reduced to nitrite, by nitrate reductase (NR), then nitrite is reduced to ammonium by nitrite reductase (NiR). Ammonium is converted into organic N compounds by the glutamine synthetase-glutamate

synthase (GS-GOGAT) pathway, producing Gln and Glu and allowing N to be incorporated into other amino acids (Crawford and Glass, 1998, Forde, 2000).
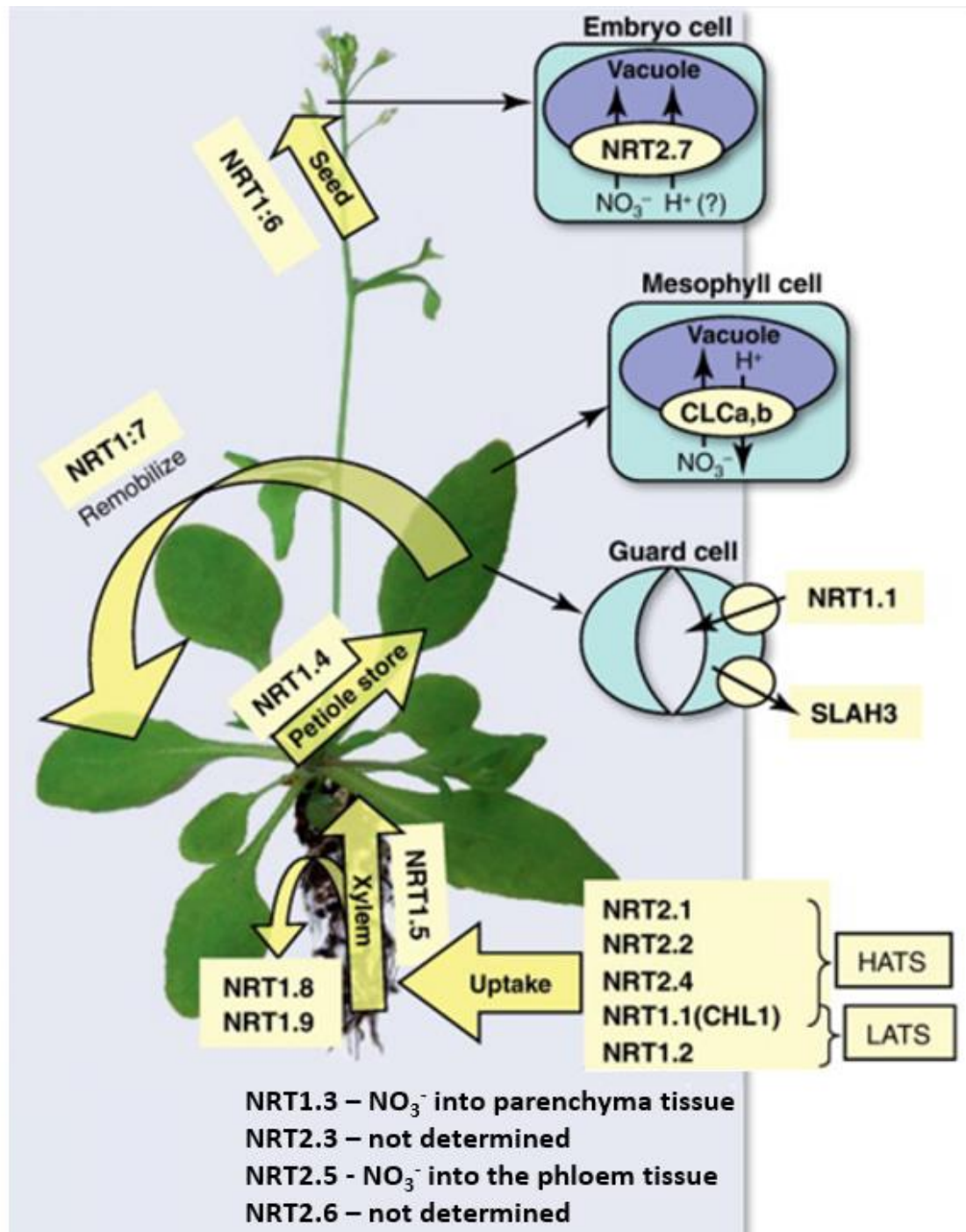
Figure 1.2 NRT1 and NRT2 transporters in Arabidopsis showing uptake of nitrate into the roots and its transportation around the plant. Figure adapted from Wang et al. (2012).

1.4.3 The transcriptional control of *NRT2.1*

The first higher plant *NRT2.1* cDNA sequence was cloned in barley (Trueman et al., 1996). The barley root library yielded two sequences, BCH1 and BCH2 which were homologous to nitrate transporter genes previously found in other organisms, specifically *Aspergillus nidulans* and *Chlamydomonas reinhardtii*. Analysis by Northern and Southern blots demonstrated that the genes were nitrate inducible and were part of a bigger gene family in barley. These findings suggested that the BCH sequences belonged to a gene family responsible for nitrate transport (Trueman et al., 1996). Filleur and Daniel-Vedele (1999) were among the first to isolate and characterise, at the transcriptional level an *NRT2.1* cDNA in an Arabidopsis background. It was reported that nitrate positively regulated *AtNRT2.1* gene transcription, which caused the AtNRT2.1 protein to actively transport nitrate into the plant's roots. This showed that AtNRT2.1 was under the transcriptional control of its encoding gene, *AtNRT2.1* (Krapp et al., 1998, Orsel et al., 2002). In the presence of prolonged nitrate treatments *AtNRT2.1* was repressed, which was not present in plants that were also nitrate treated, but then transferred to N free media. It was hypothesised that this repression of *AtNRT2.1* was due to the accumulation of amino acids, produced as end products of the N assimilation pathway (Filleur and Daniel-Vedele, 1999). The presence of N repression of NRT2.1 was observed early on, but only partially understood. Experiments using *Lemna* documented this induction and repression pattern explaining that the rate of nitrate influx into the roots is negatively correlated to the accumulation of internal nitrate (Ingemarsson, 1987, Doddema et al., 1976). This relationship was clarified when the exogenous application of amino acids was used to raise the concentration of internal amino acid pools, resulting in the down-regulation of *NRT2.1*, and subsequently a decrease of nitrate uptake (Krapp et al., 1998, Muller and Touraine, 1992). This suggests that it is the internal high nitrate concentration being reduced into amino acids which causes repression of *NRT2.1*. These finding were further supported through the use of NR mutants which, when supplied with nitrate, showed *NRT2.1* induction, in the absence of repression. It was hypothesised that, as nitrate could not be reduced *NRT2.1* expression remained high, with amino acid accumulation not occurring to cause feedback repression (Filleur and Daniel-Vedele, 1999). Additionally, the use of *AtNrt2.1* mutants, under nitrate inducible conditions did

not display an upregulation of HATS activity, yet they showed no difference in the LATS response to the nitrate treatment (Filleur et al., 2001).

Zhuo et al. (1999) also reported that amino acids are effective at repressing *AtNRT2.1* expression, using various inhibitors of N assimilation to promote the accumulation of amino acids in Arabidopsis. Inhibitors used in this study included L-methionine sulfoximine, tungstate and a combination of aminooxyacetate and azaserine, to prevent the conversion of ammonium to Gln, inhibit NR activity and inhibit aspartate aminotransferase and GOGAT, respectively. The use of each inhibitor resulted in a significant repression of *AtNRT2.1* expression, suggesting that amino acids produced in the N assimilation pathway are involved in N repression (Zhuo et al., 1999, Lejay et al., 1999). A study in barley also reported similar inductive effects for nitrate and repressive effects of amino acids applied in the presence of a nitrate induction treatment on *HvNRT2.1* expression (Vidmar et al., 2000). The exogeneous application of nitrate induced *HvNRT2.1* expression, while separate amino acids (Asn, Asp, Gln, Glu) applied in the presence of a nitrate induction treatment resulted in the significant repression of *HvNRT2.1* expression. In addition, the study also used chemical inhibitors of N assimilation to increase internal concentrations of specific amino acids. These inhibitors were tungstate, methionine sulfoximine, and azaserine, applied separately to inhibit nitrate reductase, glutamine synthetase, and glutamate synthase, respectively. All inhibitors caused a significant repression of the *HvNRT2.1* transcript, showing that Gln is among the most influential amino acids in down regulating *HvNRT2.1* (Vidmar et al., 2000a). Northern blot analysis of Arabidopsis seedlings treated individually with major amino acids involved in the N assimilation pathway (Asn, Asp, Gln, Ala, Glu) also showed that Gln was the most effective at repressing *AtNRT2.1* (Nazoa et al., 2003). Additionally, levels of each amino acid in the phloem, after exogenously applying these amino acids to Arabidopsis roots were measured. Gln application not only produced the highest concentrations of the different amino acids in the phloem, but it was also the amino acid in the greatest abundance, regardless of the amino acid treatment (Nazoa et al., 2003). It is possible that the application of Gln has such a strong repressive effect on *NRT2.1* as it promotes an increased concentration of other amino acids in internal N

pools. However, its high abundance in all treatments suggests it has a major role in the N signalling pathway.

Growing Arabidopsis seedlings in the presence of high concentrations of N (10 mM nitrate) was reported to repress *NRT2.1* in Arabidopsis seedlings (Cerezo et al., 2001). However, when these seedlings were transferred to N–free media, a period of N starvation resulted in the derepression of *NRT2.1*, consequently restoring activity of the HATS. It is hypothesised that as the internal amino acid pools are utilised and as no additional nitrate can be reduced, *NRT2.1* is no longer repressed (Cerezo et al., 2001, Filleur and Daniel-Vedele, 1999). This supports the role of amino acids in the repression of *NRT2.1*. To highlight the importance of NRT2.1 on HATS activity an *Atnrt2.1* Arabidopsis mutant was subjected to an N starvation treatment, showing that remaining HATS activity was not nitrate inducible, or repressible by ammonium nitrate (Cerezo et al., 2001). This helped to demonstrate the important role of NRT2.1 as the inducible regulator of the HATS.

While, in general, low concentrations of nitrate and high concentrations of amino acids induce and repress *NRT2.1* expression, respectively, certain conditions were reported to alter this response. The application of ammonium or Gln ≥1 mM, in the presence of ≤0.5 mM nitrate did not repress *NRT2.1* expression (Krouk et al., 2006). It was suggested that the ammonium used in this experiment repressed NRT1.1 function, a known upstream regulator of *NRT2.1* expression allowing it to be induced by the low concentration of nitrate. Additionally, it was proposed that this upregulation of *NRT2.1*, and in turn nitrate transport could be to counter the high intake of ammonium, known to be toxic to the plant (Krouk et al., 2006). However, increasing the ammonium concentration resulted in the repression of *NRT2.1* despite the presence of low concentrations of nitrate (Krouk et al., 2006). These results suggest that NRT1.1 has a powerful regulatory effect upon *NRT2.1* expression.

A sequence upstream of the TATA box within the promoter region, specifically 150 bp long was identified which is required for positive and negative regulation of *NRT2.1* by nitrate and amino acids, respectively (Girin et al. 2007). This was reported in an Arabidopsis reporter line containing a 1201 bp fragment corresponding to the sequence located upstream of the initiating codon of *AtNRT2,* fused to a β-glucuronidase (GUS) reporter gene

(*pAtNRT2.1(1201)::GUS*). GUS activity was shown to be repressed under high N conditions, separately with 5 – 20 mM nitrate and 10 mM ammonium nitrate. The application of 10 mM ammonium and 10 mM amino acids treatments, including Gln and Asn was also shown to drastically lower GUS expression in *pAtNRT2.1(1201)::GUS.* It was also observed that sucrose upregulated both the *NRT2.1* transcript and GUS activity, in conjunction with the findings of Lejay et al. (1999). This confirms that in addition to being regulated by N sources *NRT2.1* expression is also influenced at the transcriptional level by the carbon status of the plant (Girin et al., 2007). The Arabidopsis *pAtNRT2.1(1201)::GUS* line was also used to show that the location of *NRT2.1* expression was both tissue specific and dependant on the plant's developmental stage (Nazoa et al., 2003). Predominantly *NRT2.1*, when induced is expressed in the roots, but some expression is present in the shoots. Mature Arabidopsis plants (3-4 weeks old) express *NRT2.1* strongly in older parts of the root, lesser in the younger tissue and possibly not at all in the tips of both the primary and lateral roots based on the GUS expression results. Younger and older plants displayed much reduced levels of GUS expression (Nazoa et al., 2003), which coincided with nitrate uptake patterns for cowpea, green gram and soybean seedlings in a separate study (Imsande and Edwards, 1988). GUS expression in the shoots showed some spotting at the base of the rosette and on some of the leaves, including the leaf tip areas (Nazoa et al., 2003). Cross sections of roots showed that *NRT2*.1 expression was on the outer cell layer, (e.g. the epidermis and the outer layer of the root hairs) (Trueman et al., 1996), but not on inner cells of the roots, corresponding with what is known about its role in uptake of nitrate from its surrounds into the roots. This experiment also reported that *NRT2.1* expression was successfully repressed with the use of amino acids, including Gln. Together the results demonstrate that *NRT2.1* is localised effectively for root nitrate uptake. *NRT2.1* not being expressed at the root tips has led to the hypothesis that another transporter is responsible for mineral N uptake at that location (Nazoa et al., 2003).

An Arabidopsis luciferase reporter line (*pNRT2.1::LUC*) containing a 1201 bp fragment corresponding to the sequence located upstream of the initiating codon of *AtNRT2.1, fused* to a firefly luciferase gene (LUC) was also used to investigate the *NRT2.1* response to different forms of N (Girin et al., 2010). The

pNRT2.1::LUC line, grown continuously under low nitrate (0.3 mM KNO_3) and high nitrate conditions (10 mM NH_4NO_3) showed that luminescence expression and endogenous *NRT2.1* expression was strongly induced and repressed, respectively. This reporter line was then subjected to an ethyl methanesulfonate mutagenisis screen in the presence of high N conditions, screening for an increase luminescence phenotype. Three mutants were identified and termed High Nitrogen Insensitive (HNI) mutants (*hni140-1*, *hni48*-1, *hni9-1*). These novel signalling mutants have been shown to be impaired in the systemic control of *NRT2.1* expression by the Arabidopsis plant's N status (Girin *et al* 2010). The study demonstrated that the mutants, each defective in different and new parts of the *NRT2.1* promoter sequence were largely insensitive to high N repression. Each mutant was analysed for total N content and no significant differences were noted, suggesting the phenotype is a signalling impairment, rather than a nutrient uptake impairment. However, the HNI mutants did contain different ratios of internal amino acids and sugars. This suggests that the modification of *NRT2.1* as a regulator gene could be responsible for the alterations in internal amino acid pool composition, as seen for different regulator genes in other studies (Girin et al., 2007, Peng et al., 2007, Gao et al., 2008). Girin et al. (2010) proposed that as the composition of metabolite pools was altered with the alteration of the regulatory gene then maybe the composition of amino acids and sugars itself are involved in regulating the pathway (Girin et al., 2007).

1.4.4 Post-transcriptional control of *NRT2.1*

Regulation at the post-transcriptional level was suggested by early studies on *NRT2.1*, but provided little evidence. Initially, it was thought that reduced forms of N could affect NRT2.1 at the protein level. Fraisier et al. (2000) was the first study to truly demonstrate that the transcript level was not a limiting factor. They showed that, even when *NRT2.1* is overexpressed under the control of a 35-S promoter, ammonium applied exogenously reduced nitrate uptake despite a high *NRT2.1* transcript accumulation (Fraisier et al., 2000). It was hypothesised that ammonium may cause changes in NRT2.1 protein activity through phosphorylation/ dephosphorylation, however this was not conclusively established (Rawat et al., 1999, Fraisier et al., 2000, Orsel et al., 2002, Forde, 2000). Ammonium post-transcriptionally regulating NRT2.1 was also proposed in

experiments where plants exposed to ammonium displayed a decrease in nitrate transport independent of the *NRT2.1* transcript (Vidmar et al., 2000). Given that the function of the protein and the transcript of *NRT2*.1 are no longer correlated it suggests that a post-transcriptional factor is regulating NRT2.1. Another study of the NRT2.1 protein found numerous conserved protein kinase C recognition motifs in the C terminal domain of NRT2.1 transporter proteins, which could be the target of ammonium post-transcriptional regulation (Forde, 2000). These motifs could be used to post-transcriptionally regulate NRT2.1 via phosphorylation/ dephosphorylation, similarly to NRT1.1 which could explain the sudden change in transporter activity observed when plants are exposed to ammonium (Forde, 2000, Orsel et al., 2002, Glass et al., 2002). Other studies suggested that nitrate itself was regulating *NRT2.1* post-transcriptionally. Observations in barley reported the upregulation of *NRT2.1* transcript levels when the reduction of nitrate to nitrite was inhibited using tungstate (Vidmar et al., 2000). However, it is more likely that in the absence of reduced forms of nitrate, there is no repressive signal to downregulate the inductive effect of accumulated nitrate.

Further studies in barley showed evidence of post-transcriptional regulation of NRT2.1. Tong et al. (2005) found that the transcription of the *Nar2* gene was essential for the N regulation of the NRT2.1 transporter. This was confirmed by Orsel et al. (2006) who reported in Arabidopsis that the *Nar2* gene encodes a partner protein to the NRT2.1 transporter (Nar2), and was required for NRT2.1 regulation. Using knockout mutants of one or both *Nar2* partner protein genes they demonstrated its necessity for the regulation of the HATS functionality of uptake at low nitrate concentrations (Orsel et al., 2006). Ishikawa et al. (2009), using immunochemical techniques showed that both the Nar2 and NRT2.1 proteins were localised at the same points in the cells. The use of recombinant proteins in an affinity binding column allowed them to identify a potential binding site for Nar2 on the NRT2.1 protein. The assay indicated that the Ser463 on the C-terminus of the NRT2.1 protein has a major role in the binding with Nar2 (Ishikawa et al., 2009). Shortly after, Yong et al (2010) suggested that Nar2.1 post-transcriptionally regulates NRT2.1 function by forming a heterooligomer complex together by presenting an increase and decrease in the molecular masses of the NRT2.1 complex at different stages of regulation (Yong et al.,

2010). These findings strongly suggest a post-transcriptional interplay between the NRT2.1 and Nar2 proteins.

Post-transcriptional regulation was further supported by Laugier et al. (2012) using a transgenic Arabidopsis *35-S::NRT2.1* line they reported that function of NRT2.1 is down-regulated under N repression conditions, despite the mutant having a high level of *NRT2.1* expression (Laugier et al., 2012). This change was also accompanied by a decrease in NRT2.1 protein abundance. It was also observed that NAR2.1 abundance was closely correlated with that of NRT2.1 (Laugier et al., 2012), suggesting that NAR2.1 was not the candidate producing this post-transcription modification. Other research suggests that NRT2.1 exists in various forms due to post-transcriptional regulation. Using an Arabidopsis *NRT2.1::GFP* reporter line, Wirth et al. (2007) reported that a variation of NRT2.1 protein forms existed, showing that inductive and repressive treatments had an immediate effect at the transcriptional level, but a much delayed one at the protein level. This lag in enzyme activity suggests that regulation of NRT2.1 is occurring post-transcriptionally at the protein level (Wirth et al., 2007). It was observed that free GFP within the plant cells, which was confirmed not to be the NRT2.1 protein was being transported to the vacuole for deconstruction (Wirth et al., 2007). This suggests that post-translational modification taking place could be involved in the partial proteolysis of NRT2.1 at the C terminal end of the protein.

The Ynt1 protein in yeast (*Hansenula polymorpha)*, which is homologous to NRT2.1, was shown to undergo ubiquitinylation upon the exogenous application of Gln to the cells. Ynt1 is then mobilised to the vacuole and degraded by enzymes (Navarro et al., 2006). This post-transcriptional mechanism supports a similar interaction that could occur for NRT2.1. It was subsequently shown that Ynt1 is supressed by reduced forms of N and that a partner protein plays a key role in its functionality (Navarro et al., 2008). This partner protein, the nitrogen permease reactivator 1 kinase (Npr1) targets the Ser-246 on the intracellular loop of the Ynt1 transporter and phosphorylates it. Phosphorylation of Ynt1 was reported to occur under N limiting conditions and it was shown that when phosphorylation could not occur the supply of nitrate no longer triggered a rapid induction of NRT2.1 transport (Navarro et al., 2008). This post-transcriptional control is so important as it is responsible for the Ynt1 promotor accumulating at the plasma membrane of the cell. Without it the Ynt1 protein was reported to

undergo increased amounts of ubiquitinoylation which leads to it being trafficked to the vacuole, for degradation (Navarro et al., 2008). These findings for Ynt1 suggest a similar mechanism may be present for the homologuous protein NRT2.1 in plants.

Widiez et al. (2011) also reported evidence of post-transcriptional regulation. Using the *hni9-1* Arabidopsis signalling mutant, which is impaired in the high N response found that the genomic mutation was allelic to a sequence involved in feedback repression of *NRT2.1*. This sequence, termed Interact With SPT6 (*AtIWSI*) regulates hundreds of genes in the plant and produces a protein involved in the makeup of RNA polymerase II (Widiez et al., 2011). In yeast, IWS1 was shown to influence chromatin remodelling at a specific gene locus under various sucrose conditions (Fischbeck et al., 2002, McDonald et al., 2010). Arabidopsis contains a conserved homolog of STP6, which interacts with IWSI, suggesting a possible interaction that could be affecting *NRT2.1* post-transcriptionally. In a *hni9/AtIWS1* mutant *NRT2.1* was not repressed under high N conditions, reportedly due to a failure to repress the cis-acting elements of the *NRT2.1* promoter. It was proposed that post-transcriptional histone modifications and chromatin remodelling, mediated by IWS1, known to regulate nutrient signalling related gene expression could be the cause of this response (Widiez et al., 2011). Their results show in the absence of AtIWS, shown to promote methylation of a histone (H3K27me3) found on specific N-responsive loci, that H3K27me3 abundance was correlated with the plants insensitive *NRT2.1* response to high N (Widiez et al., 2011). These finding suggests yet another method of regulation at the post-transcriptional level, shedding more light on the complexity of N-signalling.

1.4.5 Long distance signalling involving *NRT2.1*

Whilst local signalling has been shown to be important, research on long distance signalling has revealed regulation also occurs systemically. Cooper and Clarkson (1989), using split root experiments in barley demonstrated that nitrate uptake into the roots was markedly increased in roots on N-sufficient media when the rest of the plant's root system was N-deprived. This was rationalised as a method of compensation based on the overall N status of the plant (Cooper and Clarkson, 1989). This concept was linked to a similar model proposed for potassium uptake

and regulation in plants, with the notion that cycling of N between the shoots and the roots could effectively form a single pool of amino acids in the plant that regulates *NRT2.1* function (Cooper and Clarkson, 1989, Siddiqi and Glass, 1987, Drew and Saker, 1984)

Tillard et al. (1998) using split-root experiments in *Ricinus communis* reported that roots in N-free conditions could increase nitrate uptake of roots in N-sufficient conditions. This suggests that a shoot-derived signal, produced by the N-starved portion of the root system was able to override the N-sufficiency signal in the nitrate treated roots. It was also reported that phloem amino acids were preferentially directed to the portion of the split root system in nitrate sufficient conditions (Tillard et al., 1998). While amino acids are associated with the repression of nitrate uptake, this suggests a long distance signalling mechanism associated with the phloem transfer of N-assimilates.

NRT2.1 was the first gene confirmed to be subject to regulation by long distance N signalling (Gansel et al., 2001). This was reported in Arabidopsis split root experiments where the *NRT2.1* transcript was increased, along with nitrate influx in roots treated with nitrate, in response to the rest of the root system being N-starved. This response suggests that long distance signals are being sent from other parts of the root system stimulating uptake activity to compensate for the roots system's spatially dependent N-deprivation (Gansel et al., 2001). Other split root research in Arabidopsis using an *Atnrt2.1* mutant showed that roots, in nitrate sufficient conditions no longer increased nitrate uptake in response to the rest of the root system being subject to N-starvation. Additionally, the application of reduced N metabolites also did not reduce nitrate uptake, respectively (Cerezo et al., 2001). While it is known that *NRT2.1* is in the major inducible component of the *NRT2* genes, these results suggest that the internal pool of amino acids in the plant could be communicating with *NRT2.1* to alter its uptake functioning.

Long distance signalling has also been reported in split root experiments using *Medicago truncatula* where roots treated with nitrate displayed an increased uptake rate in response to the rest of the root system being N-starved. This response was correlated to an upregulation of *NRT2.1* expression in the treated roots. However, when the experiment was performed using ammonium nitrate an increase in N uptake was not observed, and *NRT2.1* expression was not upregulated. While these amino acids have different uptake systems this

demonstrates that long distance signalling is not simply one pathway dependent on the N status of the plant, it is rather a number of pathways each of which are N-source specific (Ruffel et al., 2008, Liu et al., 2009). Evidence for other macronutrient sources regulating *NRT2.1* and nitrate uptake has also emerged, with shoot-derived carbohydrate signals behaving in a similar manner to that of phosphorus long distance signalling (Hammond and White, 2008). It was shown that the expression of the nitrate transporters, *NRT1.1* and *NRT2.1* were both diurnally regulated and strongly stimulated by sugar sources (Lejay et al., 2003), suggesting that the carbohydrates produced via photosynthesis in the leaves and transported to the roots in the phloem are also influential.

Studies have reported that Glu could have an important role as a long-distance signalling molecule in plants (Forde et al., 2013, Miller et al., 2007, Forde and Lea, 2007). In Arabidopsis there are 20 Glutamate-Like Receptor (*GLR*) genes that encode amino acid gated calcium ion channels shown to contain all the essential genetic domains as iGluRs in mammalian cells (Davenport, 2002). In mammals these channels interact with L-Glu and form an important signalling aspect of the central nervous system (Forde and Lea, 2007, Davenport, 2002). A range of amino acids, including Glu are known to interact with GLRs and play a role in the signalling response of plant defence (Kwaaitaal et al., 2011). However, the relationship between amino acids and GLRs has yet to be implicated in N signalling (Forde and Lea, 2007, Lam et al., 2006).

Using the *pNRT2.1(1201)::GUS* reporter line in a split root experiment Girin et al. (2007) showed that a strong nitrate induction of *NRT2.1* in low nitrate conditions could be repressed by exposing the rest of the root system to a high ammonium nitrate concentration (Girin *et al* 2007). This provides further support for the evidence of long distance signalling regulating *NRT2.1*. Girin et al. (2010) with the Arabidopsis high nitrogen insensitive (HNI) mutants (*hni9-1*, *hni48-1*, *hni140-1*) presented a new class of signalling mutant which is not regulated by N long distance signalling. This was demonstrated in split root experiments where the mutants roots in low nitrate conditions displayed increased *NRT2.1* expression in response to the rest of the root system being exposed to high N conditions (Girin et al., 2010). Comparatively, in the wild-type plants no increase in *NRT2.1* expression was present, showing that the mutants are insensitive to long distance signalling, potentially elicited by reduced amino acids. Further

analysis has shown that in the *hni9-1* and *hni48-1* lines displayed lower concentrations of internal Gln, compared to wild-type Arabidopsis (Girin et al., 2010), suggesting that the regulation of *NRT2.1* and other stages in the N assimilation pathway are altered in the mutants.

Recent developments in long distance signalling of N acquisition in Arabidopsis reported that a bZIP transcription factor involved in the regulation of shoot growth in response to light, Elongated Hypocotyl 5 (HY5) were observed to regulate *AtNRT2.1* induction in response to nitrate (Chen et al., 2016). HY5 in the shoots promotes the production of carbohydrates and sugars from photosynthesis and increases their transport to the roots, which in turn upregulates *AtNRT2.1*. However, using a *HY5-GFP* line it was reported that HY5 was translocated to the shoots in response to nitrate, suggesting that HY5 itself is a signalling molecule. In *hy5* Arabidopsis mutants the light and sugar induction of *NRT2.1* expression did not occur, demonstrating its important role in *NRT2.1* regulation. Conclusively, they reported that HY5 directly interacts with *NRT2.1,* using ChIP and EMSA to show that HY5 binds to the *NRT2.1* promoter, allowing positive regulation of *NRT2.1* (Chen et al., 2016).

Recent research has reported that a mobile hormone in Arabidopsis, C-terminally encoded peptide (CEP) has a role in root to shoot signalling of *NRT2.1* regulation (Ohkubo et al., 2017). Split root Arabidopsis experiments reported that N-starved roots induced the production of CEP, which is detected by a leucine-rich repeat receptor kinase, CEP Receptor 1 (CEPR1) found in the leaves. CEPR1 induces the production of CEP Downstream 1 (CEPD1) and CEP Downstream 2 (CEPD2) which are transported to the roots in the phloem and reported to regulate the induction of *NRT2.1* by nitrate. This was shown using a *cepr1-1* mutant in the presence of nitrate which failed to upregulate *NRT2.1*. Additionally, they overexpressed *CEPR1,* fusing it to a 35-S promoter and reported that *NRT2.1* induction in the presence of nitrate was increased up to 7-fold, compared to the wild-type (Ohkubo et al., 2017). These findings suggest an important role for CEP in regulating *NRT2.1* expression.

1.4.6 Summary of *NRT2.1* and NRT2.1 regulation

As described in this literature review there are many regulatory factors that influence the expression of *NRT2.1* and the activity levels of NRT2.1. These

regulatory factors have been shown to be responsive to the N status of the plant and are under the influence of local and long distance signalling. The *NRT2.1* gene, and consequently the NRT2.1 transporter responses to nitrate can be categorised in a number of stages for Arabidopsis (Fig. 1.3): 1. Nitrate available to the plant roots is passively imported by members of the NRT1 and NRT2 families. 2. Imported nitrate in the root upregulates *AtNRT2.1* expression. 3. NRT2.1 transporter activity increases, as its activity is under the transcriptional control of its gene, causing nitrate to be actively imported into the roots. 4. Internal nitrate is reduced in the N assimilation pathway, resulting in the production of reduced forms of N (i.e. amino acids, such as Gln and Glu). 5. As internal nitrate concentrations are progressively reduced end products of assimilation accumulate inside the plant. 6. This accumulation of amino acids results in the repression of *AtNRT2.1* expression. 7. Repression of *AtNRT2.1* results in the reduction of NRT2.1 activity, causing a strong decrease in the transporter's activity. 8. Over time the plant utilises internal N stores causing the concentration of amino acids to decrease. 9. The depletion of internal amino acids pools results in the alleviation of *AtNRT2.1* repression.
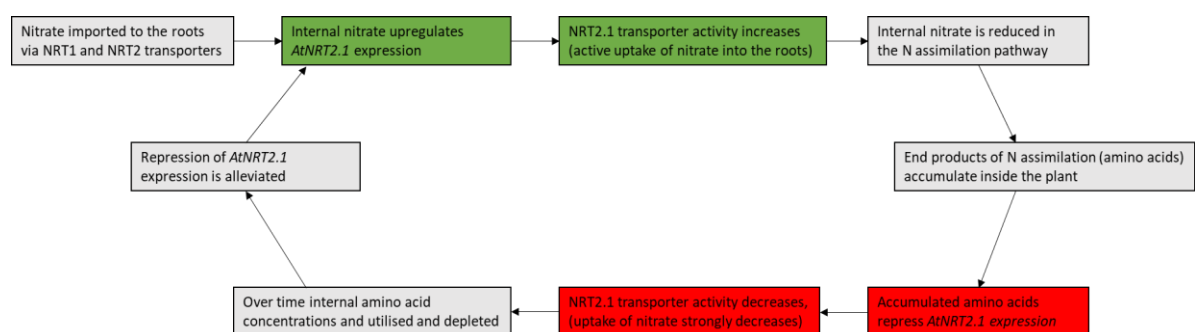
Figure 1.3 Overview of *AtNRT2.1* and NRT2.1 regulation in response to nitrate in Arabidopsis Gene expression or protein activity is either upregulated (green) or repressed (red) based on the N status of the plant.

1.5 The importance of chemical biology

The use of chemical genetics has emerged in recent years as a powerful new tool to probe plant signalling pathways, more specifically their corresponding

genes in a high-throughput manner (Dejonghe and Russinova, 2017, Norambuena et al., 2009, McCourt and Desveaux, 2010). The premise behind this approach is to target a process or gene of interest, with a readily measurable signal or plant trait and challenge it with bioactive chemicals, screening for an interaction. These libraries can contain a wide range of natural and synthetically produced compounds, selected for bioactivity in specific species or targeting certain signalling pathways. The identification of novel compound interactions allows poorly understood processes to be characterised and mechanisms associated with the targeted process to be uncovered (Serrano et al., 2015, McCourt and Desveaux, 2010, Dejonghe and Russinova, 2017).

A chemical genetics approach presents certain advantages over traditional genetics. Applied chemicals can have a transient and reversible effect on a process of interest, which can temporarily produce the same effect as a loss or gain of function allele in a mutant plant line (McCourt and Desveaux, 2010). This avoids the issue of functional gene redundancy and lethal gene alterations that are associated with the production of mutant lines for a traditional genetics approach. However, it is important to note that the use of mutant lines is still prevalent in the field of chemical genetics to both validate screening assays and help confirm the target of identified chemicals of interest (Dejonghe and Russinova, 2017, McCourt and Desveaux, 2010).

Today chemical libraries contain molecules that are new to the scientific community due to novel developments in chemical synthesis techniques. These advances have given academics worldwide access to libraries that before would have only been available to industrial companies (McCourt and Desveaux, 2010). Typically, chemical libraries are distributed in small concentrated quantities in a microtiter assay plate containing 96 or 384 wells. This not only allows for easy distribution of the libraries, but a compact way to store them in a non-industrial laboratory context (McCourt and Desveaux, 2010, Norambuena et al., 2009, Dejonghe and Russinova, 2017).

1.6 Aims and objectives

The overall aim of this thesis was to adopt a chemical genetics approach to investigate the mechanism of N feedback repression on the nitrate transporter

gene *NRT2.1* in Arabidopsis. This involved: 1. Identifying a suitable Arabidopsis reporter line to monitor *NRT2.1* expression; 2. Developing a screening platform that would both accommodate the relevant reporter line assay and allow the screening of chemical libraries in a high-throughput manner. 3. Optimising the procedures associated with the developed screening platform to determine that it was statistically viable. 4. Screening thousands of small bioactive molecules in the search for antagonists of *NRT2.1* N-repression; 5. Perform a detailed analysis of small molecules identified in this screen to determine the mode of action in relation to *NRT2.1*.

This is the first report of a chemical genetics approach being used to investigate the mechanisms of N-repression. Until now the specific mechanisms of *NRT2.1* N-repression have remained undetected despite much investigation. This research moves beyond the previous studies described in the literature review as it employs thousands of bioactive chemicals, known to have targets in plants while focusing on *NRT2.1* N-repression. This provides the possibility to observe *NRT2.1* N-repression under novel conditions as, for the first time, thousands of previously untested interactions were employed to challenge the poorly understood process of N-repression.

1.7 Hypothesis

This research hypothesises that the expression of *NRT2.1* is responsive to external sources of N as they influence the N-signalling status of the plant, which in turn regulates the expression of *NRT2.1*. Through screening multiple chemical libraries containing a vast and diverse array of small bioactive molecules it is hypothesised that the broad range of interactions within the plant will yield some molecules that influence the regulatory mechanisms associated with *NRT2.1* N-repression. These chemicals are hypothesised to antagonise *NRT2.1* N-repression by interfering with the regulatory components under the influence of the N-signalling status of the plant.

Chapter 2. Materials and Methods

2.1   Plant materials

2.1.1 Wild type Arabidopsis thaliana

Wild type plants, *Arabidopsis thaliana* L. accession, (Columbia ecotype, Col-0) were obtained from the European Arabidopsis Stock Centre (catalogue no. N60000, http://arabidopsis.info/).

2.1.2 Arabidopsis nitrogen signalling reporter lines

The NRP-YFP Arabidopsis line (Columbia ecotype, Col-0) contained a synthetic promoter with fragments from *NIA1* and *NiR* joined onto a 35-S minimal promoter (Wang et al., 2009) and were obtained from Dr Nigel Crawford, University of California. The YFP signal produced by this line was used to investigate the N status of the plant.

The *pAtNRT2.1(1201)::GUS and pAtNRT2.1(546)::GUS* (Columbia ecotype, Col-0) Arabidopsis line contained a fused 1201 bp and 456 bp fragment corresponding to the sequence located upstream of the *AtNRT2.1* initiation codon to the β-glucuronidase (GUS) coding sequence (Girin et al., 2007). Promoter fragments were originally inserted at the *Nco1* restriction site, at the initiation codon of the *GUS/3' NOS* reporter gene of the pBin19 binary vector (Bevan 1984). The *pAt35-S::GUS* Arabidopsis line contained a CaMV 35S promoter fused to the GUS coding sequence (Columbia ecotype, Col-0). Seeds for both lines were obtained from Dr Marc Lepetit, French National Institute for Agricultural Research. The GUS signal produced by these lines was to investigate the expression of *NRT2.1*.

The *pAtNRT2.1::LUC* line (Columbia ecotype, Col-0) contained a 1,202 bp promoter DNA fragment, located upstream of the *NRT2.1* translation initiation codon (*HindIII-NcoI* fragment; Nazoa et al., 2003) fused to the promoterless cassette LUC::tNOS (derived from pSP-luc+, Promega). This cassette was inserted as a *HindIII-EcoRI* fragment into the pBIB-HYG binary vector (Becker, 1990; Girin et al., 2010). Seeds were obtained from Dr Marc Lepetit, French National Institute for Agricultural Research. The luminescence signal produced by this line was used to investigate the expression of *NRT2.1*.

2.1.3 Arabidopsis topoisomerase mutant lines

The *AtTop1α-1* mutant was identified in T-DNA–tagged lines, in the Arabidopsis Columbia background (Takahashi et al., 2002).

The *AtTop1α-2* mutant was identified in an ethyl methylsulfonate mutagenizing screen of an Arabidopsis *ag-10* mutant line, in the Arabidopsis Columbia background. *AtTop1α-2* contained a C-to-T mutation present in the second exon of *TOP1 α* (Liu et al., 2014).

The *Atmgo1-7* mutant line containing an insertion allele *mgo1-7* (SALK_112625) originally identified from a SALK collection of T-DNA– tagged lines, in the Arabidopsis Columbia background (Graf et al., 2010; Alonso, 2003).

All topoisomerase mutant lines were obtained from Dr Yoshibumi Komeda, The University of Tokyo and were used to investigate the primary root growth response compared to the wild-type in the presence of camptothecin.

2.1.4 High nitrogen insensitive (HNI) Arabidopsis signalling lines

The *hni140-1* and *hni48-1* lines were originally identified from an ethyl methylsulfonate mutagenizing screen performed on the *pAtNRT2.1::LUC* line in the Arabidopsis Columbia background. The mutations were mapped, the *hni140* mutant was linked to the nga1280 marker (approx. 11 cM from the mutations) and the *hni48-1* was linked to the nga111 marker (approx. 11cM from the mutation) (Girin et al., 2010). Seeds for both lines were obtained from Dr Marc Lepetit, French National Institute for Agricultural Research and were used to investigate the effect of N-repression in comparison to wild-type under this study's conditions.

2.2 Chemical libraries

2.2.1 The 'Library of AcTive Compounds on Arabidopsis' (LATCA library)

The LATCA Library (Zhao et al., 2007) consists of 3,580 compounds and was a kind gift of Dr Sean Cutler (University of California, Riverside). The chemicals were supplied in dimethyl sulphoxide (DMSO) at 2.5 mM in 96-well plates. When used for the primary screen 1.5 µl of each stock chemical was added to the liquid medium in the assay plate well (final volume 150 µl), giving an initial concentration of 25 µM of the chemical and 1% (v/v) DMSO in the well. Additional information can be found at http://www.thecutlerlab.org/2008/05/latca.html

2.2.2 The 'Library of Pharmacologically Active Compounds' (LOPAC library)

The LOPAC Library (Sigma-Aldrich, UK) consisted of 1280 compounds at 10 mM. When used for the primary screen, 1.5 µl of each stock chemical was added to 150 µl liquid medium in the assay plate well, giving an initial concentration of 100 µM of the chemical and 1% (v/v) DMSO. The composition of the LOPAC library is available from the supplier's website (http://www.sigmaaldrich.com/life-science/cell-biology/bioactive-small-molecules/lopac1280-navigator.html).

2.2.3 'The Spectrum Collection' chemical library

The Spectrum Collection (MicroSource, Discovery Systems, Inc, USA.) consisted of 2560 bioactive compounds and natural products at 10 mM in DMSO. When used for the primary screen, 1.5 µl of each stock chemical was added to 150 µl liquid medium in the assay plate well, giving an initial concentration of 100 µM of the chemical and 1% (v/v) DMSO in the well. The composition of the Spectrum Collection is available from the supplier's website (http://www.msdiscovery.com/spectrum.html).

2.3 Nutrient media

All growth media were based on Gamborg's B5 nutrient medium (Gamborg et al., 1968).

2.3.1 Concentrated Stock Solutions. The compositions of the 10 x Macronutrient and 100 x Micronutrient stock solutions are shown in Tables 2.1 and 2.2, respectively. Stock solutions were stored in the dark at 4°C for up to several months.

Table 2.1 Composition of the 10 x macronutrient stock solution and final concentrations.

Chemical	Concentration in stock solution	Final concentration in B5/50
KCl	50 mM	0.1 mM
$MgSO_4$	20 mM	0.04 mM
$CaCl_2$	10 mM	0.02 mM
$NaHPO_4$	11 mM	0.022 mM
$MnSO_4$	450 µM	0.9 µM

Table 2.2 Composition of the 100 x micronutrient stock solution, and final concentrations.

Chemical	Concentration in stock solution	Final concentration in B5/50
KI	450 µM	90 nM
H_3BO_3	4.85 µM	0.97 nM
$ZnSO_4$	0.7 µM	0.14 nM
$CuSO_4$	10 µM	2 nM
Na_2MoO_4	103 µM	20.6 nM
$CoCl_2$	10.5 µM	2.1 nM

These stock solutions were used to prepare a 20x basal medium that did not contain N or sucrose. To make the 20 x basal medium approximately 900ml of dH_2O was added to a 1 L measuring cylinder. To this the following was added (in order):

1) 40 ml 10 x Macronutrient stock solution
2) 4 ml 100 x Micronutrient stock solution
3) 0.8 ml 90 mM Fe-Na-EDTA (final concentration 3.6 mM in B5/50 solution)
4) 10 g 2-(N-Morpholino)ethanesulfonic acid, 4-Morpholineethanesulfonic acid (final concentration 2.56 mM in B5/50 solution)

The pH of the 20 x basal medium was adjusted to 5.7 using potassium hydroxide.

2.4 Seed preparation

2.4.1 Seed sterilisation

Arabidopsis seed were washed for 1 min in absolute EtOH, then 10 min in a 20% (v/v) bleach solution containing 0.01% (v/v) Triton X-100, followed by five washes with sterile dH_2O. Seed was allowed to air dry in the flow bench, except when it was to be sown wet on the screening platform, in which case it was kept in water in the dark at 4°C for 48 h for stratification.

2.5 Arabidopsis growth on vertical agar plates

Vertical agar plates (90 mm diameter, triple vented, Starstedt, www.sarstedt.com) contained 24 ml of nutrient medium (B5/50 basal medium, 20 µM Gln, 0.5% sucrose, 1% (w/v) agar). 20 seeds were sown in a line at the top of the plate using a sterile toothpick. Plates were placed in the growth room at 22°C, 16 h light/ 8 h dark photoperiod with a light intensity of 70 µmol m^{-2} s^{-1}).

2.6 Screening platform

2.6.1 The FrameStrip method This setup (Figs. 2.1 and 2.2) was based on a previously described microphenotyping platform (Forde et al., 2013). It consisted of twelve sterile FrameStrips (4titude, http://www.4ti.co.uk) each containing eight 0.3 ml growth tubes. Each tube was filled, under sterile conditions with 250 µl of nutrient medium (B5/50 basal medium with 25 µM NH_4NO_3, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% (w/v) sucrose, 0.8% (w/v) Phytagel). The bases of the tubes were excised approximately 2 mm from the base and the FrameStrips were placed in a 96- well microtiter plate (Costar, Fisher Scientific), each well containing 150 µl liquid nutrient medium (B5/50 basal medium with 25 µM NH_4NO_3, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% (w/v) sucrose). A suspension of sterile Arabidopsis seeds were pipetted onto the surface of the agar in each tube (five to six seeds per tube) and the platform was placed in a growth box (Fig. 2.3). The growth box was transferred to the growth room and seeds were allowed to germinate. Typically, after 7 days of growth the primary root would have reached the bottom of the tubes.
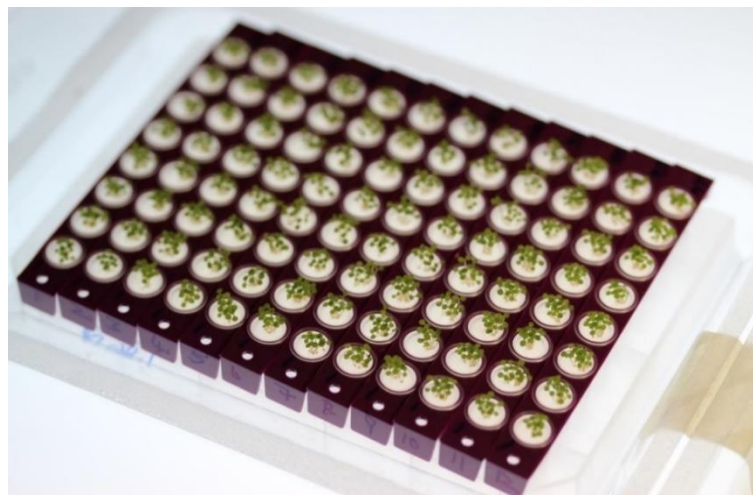
Figure 2.1 The set-up of the FrameStrip method. The typical platform set-up is shown, with twelve FrameStrips, each comprising of eight growth tubes, placed in a 96-well assay plate. Each tube contains about 300 µl nutrient medium and has been sown with five to six Arabidopsis seed.
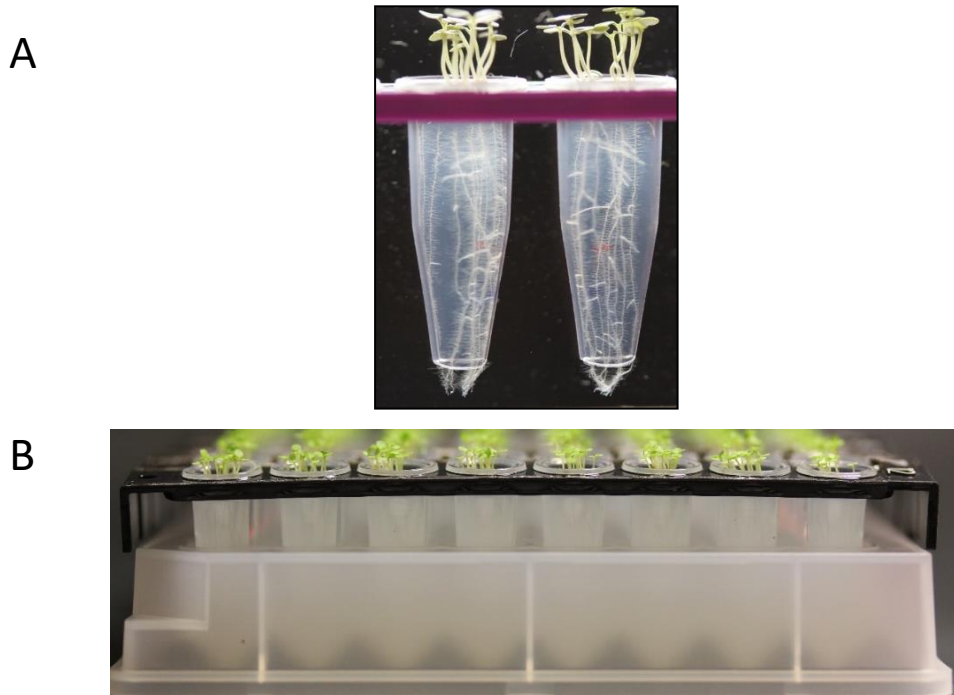
Figure 2.2 A closer look at the FrameStrip method setup: A. side view of the growth tubes filled with solid media in which the Arabidopsis seedlings were grown; **B**. a side view of the Framestrip set-up, showing growth tubes sitting in the 96-well assay plate.

Figure 2.3 Growth box housing the FrameStrip platform. Plastic packaging from the assay plates was used to create a growth box. One end is secured with tape to form a hinge, the other is secured with a paper clip. The overall dimensions are 13 cm x 9.5 cm x 5 cm.

2.6.2 The luciferase screening platform. For screens involving the *pNRT2.1::LUC* reporter line the FrameStrip method was used with some modifications to allow sufficient root material to be harvested from each tube for luciferase assays. The NH_4NO_3 concentration was increased to 50 µM and the Phytagel concentration was reduced to 0.7% (w/v). For consistency solid growth medium in growth tubes was standardised at 250 µl. Additionally, the surface of the growth tube solid nutrient media was broken up with a pipette tip prior to seed sowing to aid penetration of the roots. The size of the growth box was also increased to 14 cm x 11 cm x 11cm to increase humidity. Additionally, the number of seeds sown per well was optimised to ensure the sufficient and consistent production of root material between growth tubes. 5 seeds per growth tube resulted in the insufficient production of root material and more than 6 seeds per growth tube produced variation in the amount of root material that was able to protrude from the root base due to seedling competition (identified visually). As a result 5 – 6 seed were sown per well for experimentation. On day 7, when the roots had reached the base of the growth tubes, the FrameStrips were raised vertically by 1.6 mm using bespoke acrylic supports, placed either side of the 96-well assay plate and secured using 3M micropore tape (Fig. 2.4). At the same time the remaining liquid medium in the wells was replaced with a fresh aliquot of 150 µl liquid nutrient medium. The platform was then returned to the growth room for an additional 5 days to allow the roots to grow out of the base of the growth tubes and into the liquid medium. By day 12 sufficient root material for experimental treatments had emerged from the base of the growth tubes (Fig. 2.5). According to the experiment, N and chemical treatments were applied over the subsequent days. On day 14 roots were harvest using the 'ice capture' method (Section 2.6.3) and analysed for luminescence via a luciferase assay (Section 2.7).

2.6.3 Harvesting root material using the 'ice capture' method. A method for harvesting roots for the luciferase assay was developed that was based on the Ice-cap method (Krysan, 2004) that was originally developed for capturing root material for genotype analysis. FrameStrips with seedlings and extended root systems were transferred to a flat-bottomed black 96-well assay plate (Thermo Scientific, UK), containing 150 µl 100 mM K^+ phosphate buffer, pH 7 per well.

Acrylic supports were installed on the new assay plate (Fig. 2.4) to ensure only the emerging roots were bathed in the buffer. The set-up was then left at -30°C overnight, allowing the buffer to freeze, capturing the roots in ice (Fig. 2.5B). While the ice was still frozen Framestrips were removed, leaving the root material behind (Fig. 2.5C) and discarded, leaving the crude root extracts captured in ice in the assay plate (Fig. 2.5D). Once solutions had thawed and reached ambient temperature the luciferase assay was performed (Section 2.7).
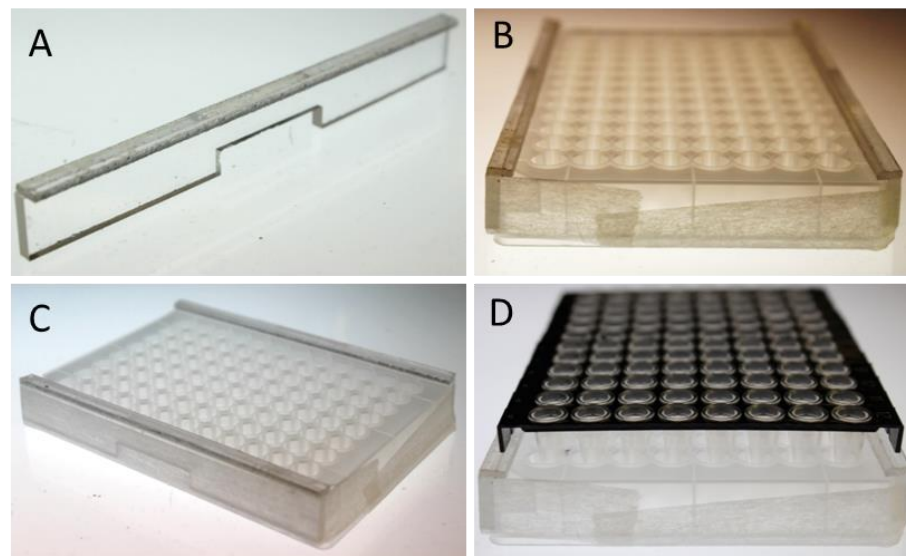
Figure 2.4 Increasing the height of the FrameStrips to allow roots to develop in the space below the growth tubes A) Each support consisted of two acrylic strips fused together: a base piece (120 mm x 3 mm x 13 mm) and a second piece (120mm x 8mm x 3mm) on top that provided a protruding lip for the FrameStrips to rest on. **B**) and **C**) Supports were installed lengthways along the assay plate. **D**) FrameStrips resting on the supports to raise the height of the growth tubes.
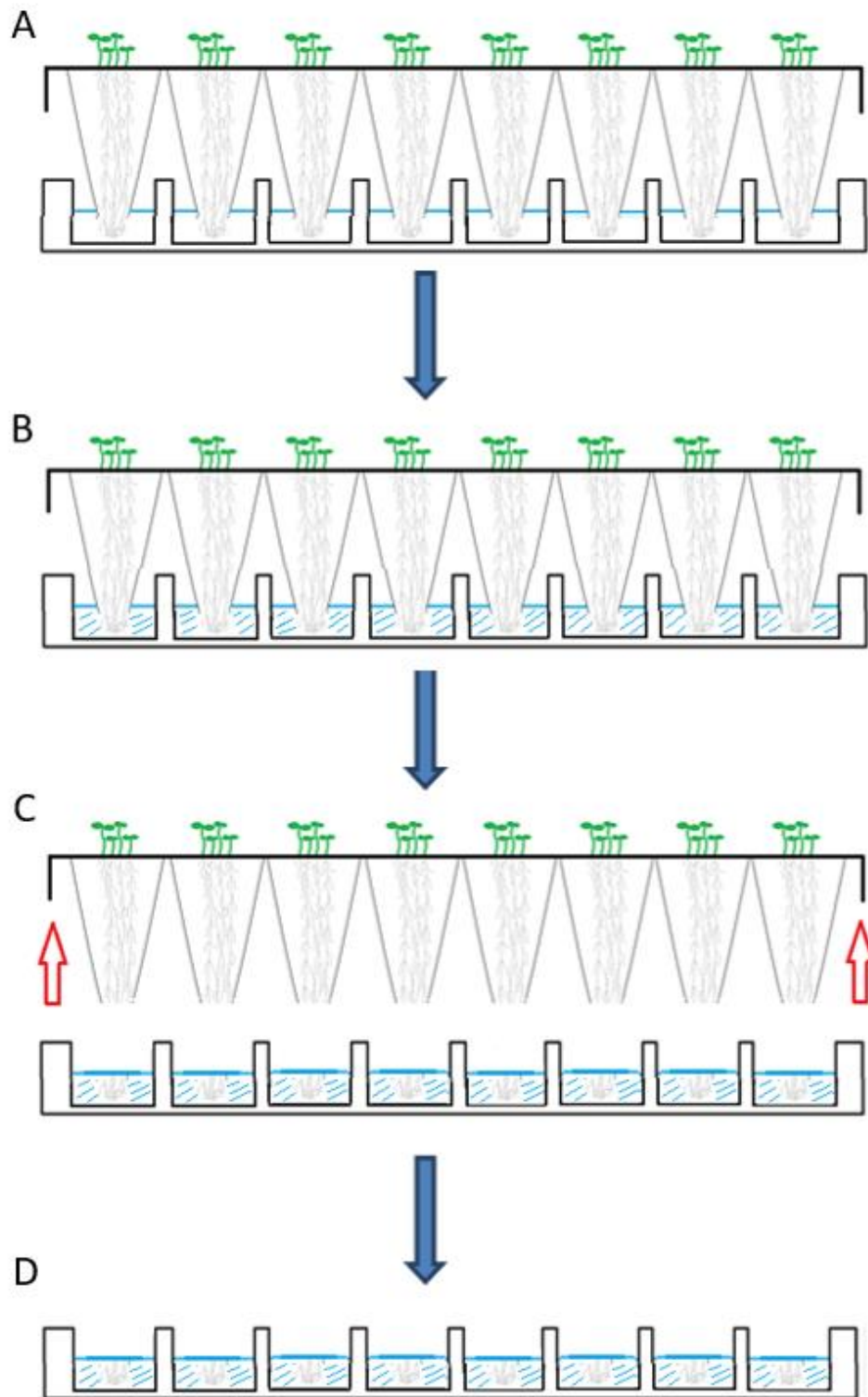
Figure 2.5 Using 'ice capture' to harvest root samples for analysis via enzymatic assay A. FrameStrips were transferred to a black 96-well assay plate where emerging root material was submerged in 100 mM K^+ phosphate buffer pH 7. **B**. The plate setup was frozen overnight, capturing the emerging root samples in ice. **C**. FrameStrips were broken off leaving root samples in the assay plate. **D**. Once the samples were thawed the crude root extracts were ready for the luciferase assay.

2.7 Assaying luciferase activity

2.7.1 Firefly luciferase bioluminescence

The luciferase assay is a favourable method for detecting interactions with a known target in the field of drug discovery (Lundin, 2000; Fan and Wood, 2007). The reaction between the luciferase enzyme, its substrate (luciferin) and ATP produces a detectable burst of light that can be used to measure gene expression in luciferase reporter plant lines, where the luciferase gene is under the transcriptional control of the gene of interest (Lundin, 2000; Baldwin, 1996; Fan and Wood, 2007). This bioluminescence reaction is used in this research to monitor the expression of *NRT2.1*, where firefly luciferase production was under the transcriptional control of the *NRT2.1* promoter.

Firefly luciferase is a 61 kDa monomeric enzyme that initially combines with luciferin to form an enzyme bound intermediate, luciferyl-AMP (a by-product of this interaction is inorganic pyrophosphate (PPI)) (Lundin, 2000; Fan and Wood, 2007). This intermediate reacts with molecular oxygen to create a high energy enzyme bound intermediate known as oxyluciferin (by-products of this interaction are H+, CO_2 and adenosine monophosphate (AMP)) (Lundin, 2000; Fan and Wood, 2007). As the high energy intermediate oxyluciferin transitions to the ground state photons are emitted, producing a burst of light with a maximum spectral range of 560 nm (Figure 2.6) (Fan and Wood, 2007).
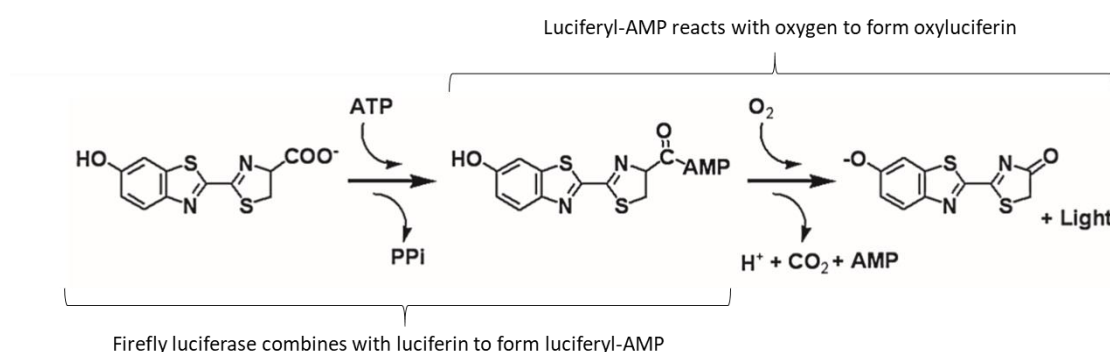
Figure 2.6 Firefly luciferase bioluminescence Molecular interactions of the firefly luciferase enzyme, the luciferin substrate and ATP in the presence of oxygen to produce bioluminescence. Diagram adapted from Fan and Wood (2007).

2.7.2 Luciferase assay

Black opaque assay plates containing crude *pNRT2.1::LUC* root extracts (produced using the FrameStrip method), 150 µl 100 mM K$^+$ phosphate buffer pH 7, 1 mM D-Luciferin, Sodium Salt (BioVision, BioVision Incoporated) and 4mM ATP (Sigma-Aldrich, St. Louis, USA), were analysed for luminescence in a dedicated luminescence microplate reader.

2.7.3 Preliminary testing for the luciferase assay

2.7.3.1 Root mass determination using propidium iodide

To ascertain that an equivalent mass of root material was being produced from each growth tube in a single luciferase screening platform setup a preliminary experiment was conducted. The screening platform was set up 8 times, using the FrameStrip method, each plate yielding 96 root samples per setup. Using the 'ice capture' method the samples were harvested into black opaque plates. Propidium iodide (10 ug/ml) was pipetted into each well manually before the plate was inserted into a microplate reader (LUMIstar Omega (BMG LABTECH)). The plate was immediately shaken (double orbital rotation, 500 rpm, 2 sec) and the fluorescence was recorded using the following settings: top down optic, emission: excitation: 535 nm, emission: 617 nm, lens height: 1 mm, measurement value: fluorescence intensity (%) using the 'Fluorescence Endpoint' read mode (read duration 5 sec). The process was repeated for each well until all the wells were processed. The microplate reader temperature was set at 30°C. Data was retrieved using MARS Data Analysis Software (BMG Labtech, UK) and transferred to Excel (data not shown). This experiment showed that there was an equivalent mass of root material between each individual sample in a single luciferase screening platform setup.

2.7.3.2 Testing the efficacy of the 'ice capture' method to release the luciferase enzyme from root material

Preliminary testing was performed to determine the effectiveness of the 'ice capture' method in releasing the luciferase enzyme from the root samples' cells. The screening platform was set up 8 times, using the FrameStrip method, with samples in columns 1-11 being induced (0.3 mM KNO$_3$, 24 hrs), while samples in column 12 were the uninduced (background N only, 200 µM NH$_4$NO$_3$). Using the 'ice capture' method the samples were harvested into black opaque plates.

The ATP was pipetted into the wells manually before inserting the plate into the luminometer. Luciferin was applied to a single well using a reagent injection system, the plate was immediately shaken (double orbital rotation, 500 rpm, 2 sec) and the luminescence recorded using the following settings: top down optic, emission: UV lens, gain: 4000 units, lens height: 1 mm, measurement value: relative light units (counts/sec) using the 'Luminescence Endpoint' read mode (read duration 5 sec). The process was repeated for each well until all the wells were processed. The luminometer temperature was set at 30°C. Data was retrieved using MARS Data Analysis Software (BMG Labtech, UK) and transferred to Excel. The experiment showed that the luminescence output was consistent within a microplate for all the induced samples, demonstrating a consistency in the 'ice capture' method's freeze-thaw process to lyse root samples' cells and release an equivalent amount of the luciferase enzyme for each sample (data not shown).

Further experimentation was conducted to determine the effectiveness of the 'ice capture' method. Repeating the same methodology, 4 screening platforms were setup, however after the 'ice capture' method, once the solutions had thawed the crude root samples were removed from the microplates (leaving only the reaction buffer the root material had been frozen in). This experiment showed a similar luminescence output to all the experiments that had included the crude root material in each well. Additionally, there was an equivalent luminescence signal produced from the well of each induced sample per plate. This demonstrated that the freeze-thaw process was effective at lysing cells of the crude root extracts, allowing the luciferase enzyme leave the root material and react during the luciferase assay in the reaction solution of each well.

2.7.4 Luminescence microplate readers

Three different microplate readers were used in the course of the project and these are described in the following sections.

2.7.5 PerkinElmer Victor II microplate reader

The ATP and luciferin were pipetted manually into the wells before inserting the plate into the luminometer. The plate was immediately shaken (double orbital rotation, 500 rpm, 2 sec) and the luminescence recorded. The settings used were:

top down optic, read mode: linear, sensitivity: extra high, measurement value: relative light units (counts/sec) using the 'Luminometry Endpoint' read mode (read duration 5 sec) for each well until all the wells were processed. The luminometer temperature was set at 30°C. Data was saved and transferred to Excel (Microsoft Office, MS).

2.7.6 LUMIstar Omega, BMG LABTECH

The ATP was pipetted into the wells manually before inserting the plate into the luminometer. Luciferin was applied using a reagent injection system to a single well, the plate was immediately shaken (double orbital rotation, 500 rpm, 2 sec) and the luminescence recorded using the following settings: top down optic, emission: UV lens, gain: 4000 units, lens height: 1 mm, measurement value: relative light units (counts/sec) using the 'Luminescence Endpoint' read mode (read duration 5 sec). The process was repeated for each well until all the wells were processed. The luminometer temperature was set at 30°C. Data was retrieved using MARS Data Analysis Software (BMG Labtech, UK) and transferred to Excel.

2.7.7 SpectraMaxi3x Multi-Mode Detection

The ATP and luciferin were pipetted into the wells manually, with the addition of coenzyme A (5 μM) before inserting the plate into the luminometer. The plate was immediately shaken (double orbital rotation, 500 rpm, 2 sec) and the luminescence recorded using the following settings: top down optic, emission: UV lens, gain: 4000 units, lens height: 1 mm, measurement value: relative light units (counts/sec) using the 'Luminescence Endpoint' read mode (read duration 5 sec). The process was repeated for each well until all the wells were processed. The luminometer temperature was set at 30°C. Data was saved and transferred to Excel.

2.7.8 Differences between the microplate readers

The use of different microplate readers throughout the course of this research revealed that the raw luminescence output measured for a single sample varied between each luminometer. Luminescence values were recorded highest with the LUMIstar Omega, BMG LABTECH microplate reader, as the on-board liquid

injection system allowed the luciferin to be applied directly to each well and read instantaneously by the machine. This allowed the luminescence reading to take at the beginning of the enzyme reaction, when activity, and subsequently luminescence output were greatest. Where the luciferin substrate was added manually (PerkinElmer Victor II microplate reader and SpectraMaxi3x Multi-Mode Detection) and then the plate was inserted into the luminometer this initial phase of the reaction was missed. After this point the luminescence output does decline over time, but it was still sufficiently strong for recording experimental data for this research (Lembert and Idahl, 1995). To control for differences between the luminometers each experiment only uses a single luminescence plate reader and the machine is specified with the data for each experiment presented in this thesis.

2.8 Treatment of *pNRT2.1::GUS* seedlings for the *GUS* histochemical assay

Seven day old *pNRT2.1::GUS* seedlings, grown on nursery plates (Section 2.5) were nitrate induced by pipetting 1 mM KNO_3 solution over their entire root system (2 ml) for a 24 h treatment period. Seedlings were then transferred to a 12-well plate (Corning®, Sigma-Aldrich), each well contained a 1 ml 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) solution (1 mM X-Gluc, 0.5 mM K^+ ferrocyanide, 0.5 mM K^+ ferricyanide, 50 mM $NaPO_4$ pH 7, 10 mM EDTA, 0.1% Triton X-100). The plate was sealed with electrical insulation tape, wrapped in the tin foil and left at 22°C, in darkness for 16 h. Plants were analysed visually using a light microscope (Leica MZFL III, Leica Biosystems) and images were captured using a CCD camera (Brunel Microscopes Ltd, UK) and transferred to the accompanying Leica software (LAS X, Leica Microsystems).

2.9 Treatment of NRP-YFP seedlings and fluorescence imaging

Seven day old NRP-YFP seedlings, grown on vertical agar plates (Section 2.5) were transferred to 12-well assay plates for a 24 h treatment with different combinations of inorganic N and Gln. Seedlings were then transferred to a glass slide and YFP expression was visualised using a fluorescence binocular microscope (Leica MZFL III, Leica Biosystems) equipped with a YFP filter (Leica Biosystems, UK) and a wide light spectrum lamp (Osram 100 W HBO mercury

short arc lamp). Excitation of YFP was via a 510 nm (20 nm band width) filter and emission was through a 560 nm (40 band pass) barrier filter. Images were acquired using ScopePhoto V3.1.475 (Brunel Microscopes Ltd).

2.10 RNA extraction and real-time PCR

2.10.1 Deep-well method for generating root material for RNA extraction

A modification of the FrameStrip method was developed to allow more root material to be generated for RNA extraction (Fig. 2.7). Experimental differences included the use of 2.5 ml deep-well V-shaped assay plates (Thermo Fisher Scientific, UK), an increase in the background N supply to 100 μM NH_4NO_3 and an increase in the volume of liquid nutrient medium to 2.3 ml per well. Seedlings were grown for 14 days, with fresh aliquots of liquid nutrient medium being supplied on day 7 and 12. Chemical treatments and N treatments were applied on days 12 and 13, respectively.
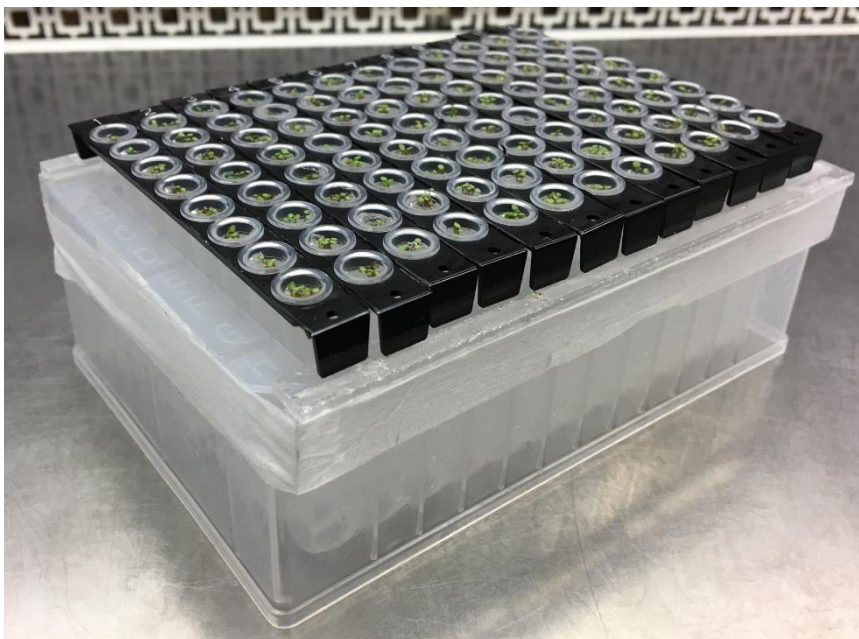
Figure 2.7 Deep-well culture of Arabidopsis seedlings for RNA extraction from roots The image shows FrameStrips in position on a deep-well plate.

2.10.2 Harvesting roots from deep-well culture

FrameStrips were removed from the assay plate, excess nutrient solution on the roots was removed with a paper towel and roots were rapidly frozen in liquid

nitrogen, in a pre-cooled mortar. Using a pre-cooled spatula, the frozen root material was severed from the FrameStrips and ground to a fine powder using a pre-cooled pestle. The frozen powder was transferred to pre-frozen 2 ml Eppendorf tubes (Sigma Aldrich, UK) and stored at -80°C.

2.10.3 RNA extraction

Total RNA was extracted and isolated from approximately 500 mg frozen root material using a modified protocol based on Verwoerd et al. (1989). The extraction was performed by adding 1 ml hot (80°C) phenol extraction and vortexing for about 30 sec until completely homogenized. The phenol extraction buffer was prepared by mixing 1 volume of phenol with 1 volume of extraction buffer [extraction buffer: 0.1 M LiCl, 1% (w/v) sodium dodecyl sulphate, 10 mM EDTA, 0.1 M Tris/HCl pH 8]. After addition of 500 μl chloroform: isoamyl alcohol (24:1) the mixture was vortexed for a further 30 sec and centrifuged at 6200 g for 5 min at 4°C. The aqueous phase was transferred to a fresh 2 ml Eppendorf tube and a second extraction performed by adding 1 ml chloroform/ isoamyl alcohol (24:1) and repeating the vortexing and centrifugation steps. The aqueous phase was transferred to a fresh 2 ml Eppendorf tube and 1 volume 4 M LiCl was added and mixed.

After allowing precipitation to occur overnight at 4°C, RNA was collected by centrifugation at 6200 g for 20 min at 4°C. Pellets were washed with 70% (v/v) ethanol and then recovered by centrifugation at 6200 g for 5 min at 4°C and allowed to air-dry. A DNase treatment was then applied by adding 7 μl of 1unit/μl RNase-free DNase, (Promega, USA), 15 μl DNase buffer (Promega, Madison, USA) and 128 μl diethyl pyrocarbonate (DEPC)-treated water. After leaving the mixture on ice for about an hour to dissolve samples were incubated for 30 min at 37°C. The volume was increased to 300 μl with DEPC-treated water and then another extraction was performed by adding 300 μl 1:1 phenol:chloroform/isoamyl alcohol, vortexing and centrifuging at 6200 g for 5 min at 4°C. The aqueous phase was transferred to a fresh 2 ml Eppendorf tube and a further extraction performed by adding 300 μl chloroform/isoamyl alcohol, vortexing and centrifuging again. The aqueous phase was transferred to a fresh 1.5 ml tube with the addition of one tenth volume 3 M NaOAc (pH 5.2) and 2.5

volumes ethanol. RNA was precipitated overnight at -30°C and collected by centrifugation at 6200 g for 20 min at 4°C. The pellet was washed with 70% (v/v) ethanol and allowed to air-dry. Finally, the RNA was dissolved in 40 to 60 µl DEPC-treated water on ice.

Total RNA concentration was measured using 1 µl of each sample using a NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, USA).

2.10.4 Gel electrophoresis

RNA quality was assessed by electrophoresis on Tris Acetate EDTA (TAE) gels containing 1% (w/v) agarose, 40 mM Tris acetate pH 8.0 and 1 mM EDTA). For loading, each sample contained 0.5 µg RNA, 2 µl 5x DNA loading buffer (Bioline, USA) in a volume of 10 µl. Gels were run at 80 V for 40 min in 1x TAE buffer and RNA staining was performed using 10 µl of SYBR Safe (Thermo Fisher Scientific, UK) per 100 ml of gel. Gel images were captured using a Gel-Doc UV viewer (Bio-Rad, CA, USA).

2.10.5 cDNA synthesis

cDNA was synthesized from total RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, USA). In a 0.5 ml PCR tube (Starlab Ltd, UK) each reaction contained 2 µg RNA, 1 µl 10 mM dT-Adapter primer (Invitrogen, UK) to a final volume of 13 µl. Samples were incubated at 70°C for 7 min to denature the RNA template and then placed on ice. To each reaction, 4 µl 5 x first strand buffer, 1 µl 0.1 M DTT, 1 µl 10 mM dNTP mix and 1 µl Superscript III Reverse Transcriptase as a mix was added, mixed and incubated in a peqSTAR 96-well Universal Gradient PCR machine (peqLAB.DE) for 5 min at 20°C followed by 2 h at 50°C. A final cycle of 15 min at 70°C terminated the reverse transcription and the synthesized cDNA was stored at -30°C.

2.10.6 Real-time PCR

In a 1.5 ml Eppendorf tube, 1.1 µl of a 1:5 dilution of cDNA sample was added to 0.6 µl 10 µM forward primer, 0.6 µl 10µM reverse primer, 0.02 µl ROX reference dye (Sigma Aldrich, USA), 8.9 µl DEPC-treated water and 11.75 µl SYBR Green Jumpstart Taq ReadyMix (Sigma Aldrich, USA). Details of the primers used are

in Table 2.3. The solution was mixed and 20 µl loaded onto a white, semi-skirted 96 well-plate (4titude, Surrey, UK). An Applied Biosystems 7500 Real Time PCR system (Life Technologies, Paisley, UK) was used to run the plates on a standard 7500 run mode (2 min at 50°C, 10 min at 95°C and 40 cycles of 1 min at 60°C) with a dissociation stage added (15 sec at 95°C, 1 min at 60°C, 15 sec at 95°C, 15 sec at 60°C). Results were visualised and analysed using the associated 7500 software, version 2.0.5. Rn values were exported and mean primer efficiencies calculated by analysing the linear phase of reaction amplification curves using LinregPCR software (Ruijter et al., 2009).

The Normalized Relative Quantification (NRQ) method (Rieu and Powers, 2009) was used to analyse the data. It was decided that the NRQ calculations were most suitable due to the use of individual primers efficiencies and normalizations within the fold change calculations. The NRQ expression was calculated in relation to the Ct values and the primer efficiency (E) of the gene target (X) and the normalizing reference gene (N): $NRQ = (E_x)^{-Ct, X} / (E_n)^{-Ct, N}$. The Ct results from the Real-time PCR analysis of genes related to N signalling under different N conditions and in the presence of camptothecin (Section 6.2.2) were normalized to Ct averages from two housekeeping genes, *AtActin2* and an uncharacterized gene (At4g26410), which were run for each batch of cDNA.

2.10.7 Real-time PCR Primers.

Table 2.3 contains information on oligonucleotide primers used in real-time PCR analysis.

Table: 2.3 Oligonucleotide primers used for real-time PCR analysis

Gene	Gene ID/ accession	Primer name	Forward primer	Reverse primer	Amplicon size	Amplification efficiency
AtActin2	At3g18780	AtActin2	CATGTTCACCACAACAGCAGAG	ATCTCCTGCTCGTAGTCAACAG	87 bp	97%
Uncharacterized protein (Kudo et al., 2016)	At4g26410	AtUKCONT	TGATTCGGCTCCAGCGATTGAG	TGGCTCCTTCCACCGATTCAAC	89 bp	95%
Nitrate transporter *AtNRT2.1*	At1g08090	AtNRT2.1	TGATTCGGCTCCAGCGATTGAG	TGGCTCCTTCCACCGATTCAAC	97 bp	92%
Nitrate transporter *AtNRT2.2*	At1g08100	AtNRT2.2	GTAAGGAGGAGCAGCAGATTGG	TTCCCTTTGTGGACGCTGCTG	99 bp	97%
Nitrate transporter *AtNPF6.3* (*NRT1.1*)	At1g12110	AtNRT1.1	TCGGCAGGTACCTAACGATTGC	TGCATCTTGGTGGTCGAAGTCC	105 bp	98%
Nitrate reductase *AtNIA1*	At1g77760	AtNIA1	CATCCATTCAACGCCGAAGCAC	TGCGGACGTAATGGAGTGGAAC	82 bp	94%
Nitrate reductase *AtNIA2*	At1g37130	AtNIA2	GGTCACGTTGAGTATCTCGGC	TATTCCGGTTCCACCTGCCAAC	96 bp	97%
Glutamine synthetase *AtGLN1-1*	At5g37600	AtGLN1-1	TGACTGACCCTTCGCAGCTAC	GATGACTTCACTGTCTTCACCAG	84 bp	99%
Glutamine synthetase *AtGLN1-2*	At1g66200	AtGLN1-2	CTGTGACCGATCCATCAAAACTTC	AGATCACTTCACTGTCTTGACCAG	99 bp	94%
Firefly Luciferase (Becker, 1990)	U47123.2	LUC	GAGCACGGAAAGACGATGACGG	TCCACAAACACAACTCCTCCGC	100 bp	98%

2.11 Statistical analysis

2.11.1 Analysis of Variance.

Analysis of Variance (ANOVA) was performed using GenStat 18th Edition Software (64-bit, VSN International Ltd). Calculations were performed to a 95% confidence of significance (F-test p value ≤0.05). Normally distributed data was analysed as it was. Data that was not normally distributed was log transformed to satisfy the assumptions of constant variance across treatment, the additivity of effects and to achieve a normal distribution. The least significant differences (LSD) of the means were calculated to a 5% level.

2.11.2 *Z* FACTOR.

The acceptability of the screening platform was assessed using the *Z* factor statistical parameter (Zhang et al., 1999) calculated using the following formula:

$$Z \text{ factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where

σ_p = standard deviation for the positive control being compared

$\underline{\sigma_n}$ = standard deviation for the negative control being compared

μ_p = mean for the positive control being compared

μ_n = mean for the negative control being compared

Z factor values greater than 0.5 are interpreted as indicating that the assay is well suited to high-throughput screening and values between 0 and 0.5 as indicating that the assay is acceptable for high-throughput screening and values below 0 as indicating the assay is unsuitable for high-throughput screening.

Chapter 3. Developing a platform for use in a chemical genetics screen for antagonists of N repression

Chemical genetics is an approach in which small bioactive molecules can be used to probe poorly understood biological processes. Chemical libraries of relevance to plant research contain compounds that can widely be grouped into plant hormones, growth regulators, herbicides and molecules of potential interest based on known biological activity in animals or yeast (e.g. pharmaceutical chemicals) (Kaschani and van der Hoorn, 2007, McCourt and Desveaux, 2010). To use a chemical genetics approach a high-throughput screening platform is required to efficiently test and identify compounds that perturb the process of interest. Plant reporter lines are a powerful tool when looking for easy ways to screen large numbers of chemicals for an interaction with a gene of interest (Burrell et al., 2017, Dejonghe and Russinova, 2017).

Like many other plant chemical genetics screens, this research will use *Arabidopsis thaliana* as the organism to screen the chemical libraries. Arabidopsis has many advantages for this approach given the many resources that are available, not least its genome sequence, the number of well-characterized mutants and reporter lines, many of which are directly related to N signalling. Additionally, Arabidopsis has a short life cycle, grows in a wide array of conditions and is relatively small in size, characteristics that favour a chemical genetics screening platform (Dejonghe and Russinova, 2017, Serrano et al., 2015, Burrell et al., 2017).

The primary objective of the work described in this chapter was to identify an Arabidopsis reporter line that would be suitable for use in a screening programme aimed at identifying chemical antagonists of N repression. Once identified, the second objective was to develop a screening platform that would be suitable for conducting large numbers of assays of the reporter line in conjunction with the application of libraries of small molecules.

The NRP-YFP Arabidopsis reporter line developed by Wang et al. (2009) was the first candidate identified for potential use in this research. It contains a synthetic nitrate-inducible promoter (NRP), composed of fragments of the *NIA1*

(nitrate reductase) and *NiR* (nitrite reductase) genes fused to a CaMV 35-S minimal promoter. Wang et al. (2009) showed that NRP was nitrate-inducible (when treated with 20 mM KNO_3), producing a significantly stronger fluorescence signal compared to uninduced seedlings (treated with 20 mM ammonium succinate). The NRP-YFP line was screened for individuals displaying reduced fluorescence under conditions of nitrate induction. Two mutants, *Mut21* (*nrg1*) and *Mut164* were found to contain mutations in the *NRT1.1* and *NLP7* genes, respectively. Comparison of *Mut21* to a well-characterised *nrt1.1* (*chl1-5)* mutant by Wang et al. (2009) showed that the expression of three nitrate-inducible genes (*NIA1*, *NiR1* and *NRT2.1*) was reduced by more than 80% in both. *Mut21* had a decreased nitrate uptake compared to the wild type, however the influx of nitrate and internal concentrations were high enough to cause *NRT1.1* induction. This showed that low fluorescence of NRP-YFP seedlings was due to regulatory changes, not a disruption of nitrate uptake (Wang et al., 2009). NRP-YFP was therefore suitable for investigating N signalling in Arabidopsis in this project.

Arabidopsis *NRT2.1* GUS fusion reporter lines, containing a fused 1201 bp or 456 bp fragment corresponding to the sequence located upstream of the *AtNRT2.1* initiation codon to the β-glucuronidase (GUS) coding sequence (*pAtNRT2.1(1201)::GUS and pAtNRT2.1(546)::GUS*) were identified as other candidate reporter lines. *NRT2.1* has been shown to be a major target of N repression, which is strongly believed to be elicited by reduced forms of N (Filleur et al., 2001, Zhuo et al., 1999, Nazoa et al., 2003). Studies using both *pNRT2.1::GUS* lines have shown that the GUS expression was induced in low nitrate conditions (0.3 mM KNO_3), repressed in high N conditions (10 mM NH_4NO_3) and correlated closely to the plants' endogenous *NRT2.1* transcriptional state (Girin et al., 2007).

An Arabidopsis *NRT2.1* luciferase fusion reporter line (*pNRT2.1::LUC)* was identified as a suitable candidate for this research. Studies using the *pNRT2.1::LUC* line demonstrated that luciferase expression was inducible under low N conditions (0.3 mM KNO_3) and was repressed under high N conditions (10 mM NH_4NO_3) (Girin et al., 2010). Luciferase expression was also strongly correlated to the endogenous expression of *NRT2.1* under these conditions (Girin et al., 2010). The study also performed a mutagenesis screen, selecting seedlings displaying increased luciferase expression under high N conditions,

termed High Nitrogen Insensitive (HNI) mutants (*hni*140-1, *hni*48-1 and *hni9-1*). It was shown that these mutants had no mutations in the *NRT2.1* promoter sequence of the LUC transgene, thus the increase in luminescence was due to an impairment in N signalling (Girin et al., 2010).

In summary, the aim of this chapter was to outline the preliminary research that was conducted in order to establish the foundations of this research project: 1) Identifying an Arabidopsis reporter line suitable for monitoring the N signalling status of the plant. 2) Establishing a physical system for growing the Arabidopsis reporter line which would be both compatible with the specific assay associated with the reporter line and was suited to the high-throughput requirements associated with chemical genetics. This was achieved by testing a range of Arabidopsis lines with reporter genes associated with the N signalling status of the plant under different N regimes. Once a suitable reporter line was selected a novel screening platform was developed around the requirements of the reporter line's screening assay that was compliant with the requirements of a chemical genetics screen.

3.1 Results

3.1.1 Investigating the Arabidopsis NRP-YFP reporter line

The NRP-YFP reporter line was first grown on vertical agar plates and induced for 24 h using 1 mM KNO_3. A strong YFP fluorescence signal was detected in roots of all induced seedlings (Fig. 3.1C), compared to the uninduced (Fig. 3.1A). NRP-YFP was then tested to see if the signal could be N repressed. When seedlings were simultaneously exposed to 1 mM KNO_3 and 5 mM Gln the YFP signal in roots was significantly reduced (Fig. 3.1B) and was visually indistinguishable from the uninduced (Fig. 3.1A).
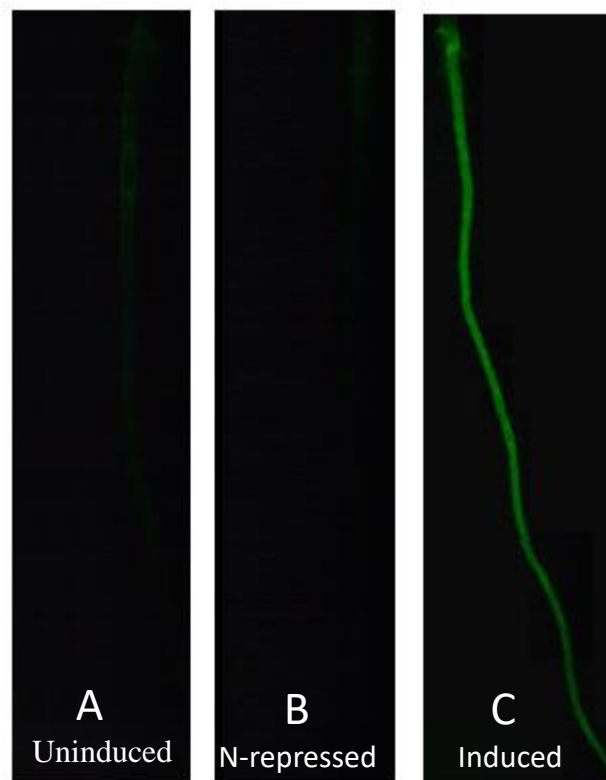
Figure 3.1 Fluorescence imaging of roots of NRP-YFP seedlings under different N regimes. Seven day old NRP-YFP seedlings, grown on vertical agar plates (see Section 2.5), were transferred to a 12-well plate and subjected to a 24 h N treatment in liquid medium (see Section 2.9). **A**. Uninduced (background N only, 20 μM NH_4NO_3). **B**. N-repressed (1 mM KNO_3 + 5 mM Gln). **C**. Induced (1 mM KNO_3). Seedlings were then placed on glass slides and imaged for YFP fluorescence using a Leica MZFL III microscope (see Section 2.9).

3.1.2 Investigating the Arabidopsis *pNRT2.1::GUS* reporter line

Seedlings of the two *pNRT2.1::GUS* lines, *pNRT2.1(546)::GUS* and *pNRT2.1(1201)::GUS,* were grown on vertical agar plates for 7 days then transferred to 12-well assay plates for treatment. Nitrate induction with 1 mM KNO_3 for 24 h produced a strong clear blue colour, predominately in the mature section of the root system (Fig. 3.2 A). To try to repress GUS expression a low (2 mM) and high (10 mM) Gln treatment were applied simultaneously with 1 mM KNO_3. Under both conditions, seedlings developed a clear blue colour predominately in the mature section of the root system (Fig. 3.2B and C). Compared to the *35S-GUS* seedlings expression was slightly less under these repressive conditions. GUS expression for all three lines was near identical when nitrate treated alone (Fig. 3.2D). When all lines were uninduced only the *35S-GUS* line developed a blue colour (Fig. 3.2A) and the Col-8 showed an absence of GUS expression under all treatments (Fig. 3.2). Of the two *NRT2.1* reporter line variants, *pNRT2.1(1201)::GUS* seedlings displayed a slightly lower level of GUS expression under N repression conditions, compared to *pNRT2.1(546)::GUS*. However, in neither case was there a very clear distinction between the degree of staining in induced or N repressed conditions.

Attempting to reduce GUS expression under N repressive conditions to a greater degree, the histochemical assay was performed for different times and at different temperatures. However, no significant improvements were produced. Additionally, a 3 h pre-treatment with Gln at both low and high concentrations before nitrate induction was tested. However no significant improvements were obtained.
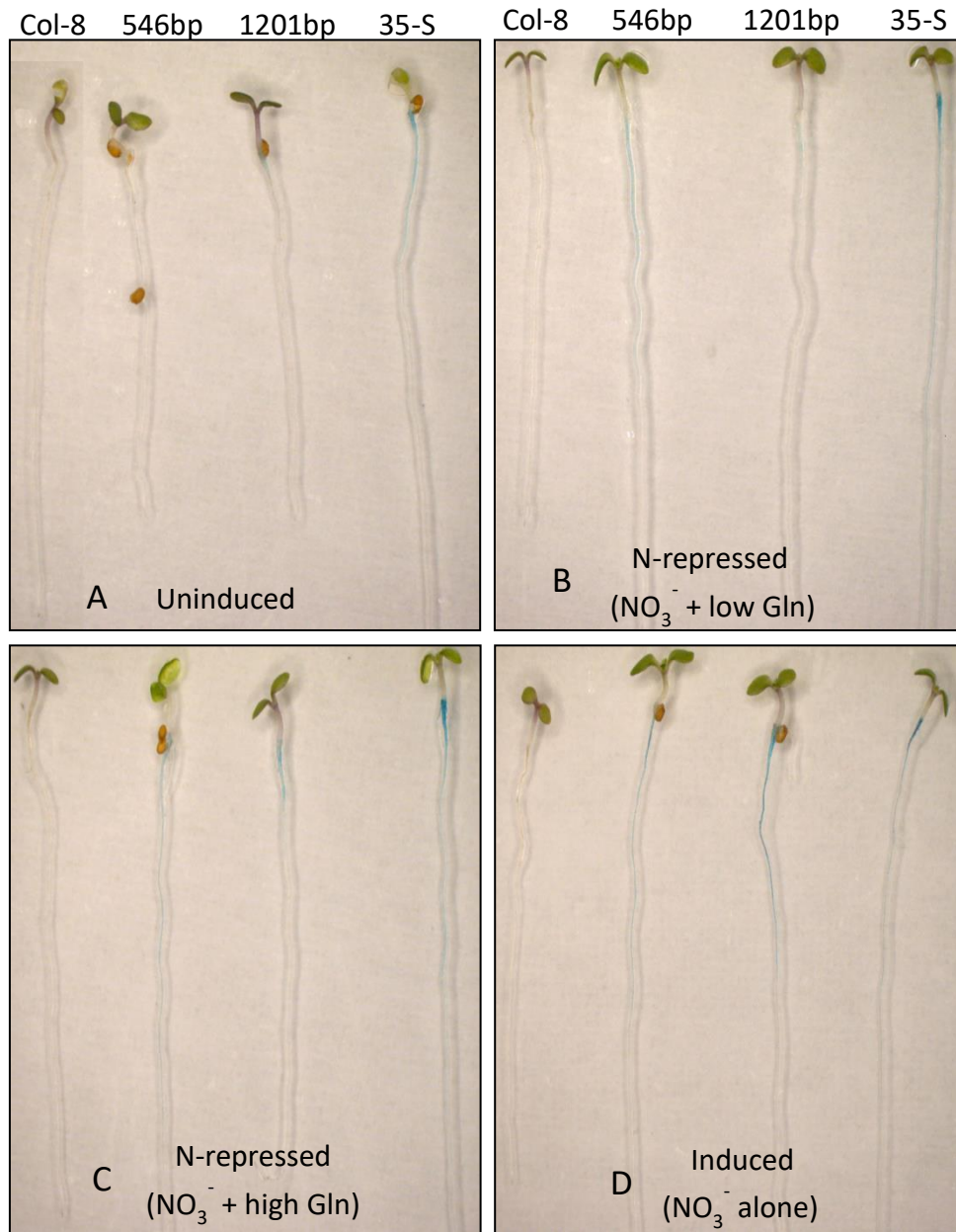
Figure 3.2 Histochemical staining of two *pNRT2.1::GUS* lines under different N regimes. Seedlings of the *pNRT2.1(546)::GUS* and *pNRT2.1(1201)::GUS* lines, along with Col-8 and a *35S-GUS* line as controls, grown on vertical agar plates were transferred to 12-well assay plates and subjected to different 24 h N treatments in the liquid medium. **A**. Uninduced (50 μM Gln, background N). **B**. N repressed, low Gln (1 mM KNO_3 + 2 mM Gln). **C**. N repressed, high Gln (1 mM KNO_3 + 10 mM Gln). **D**. Induced (1 mM KNO_3). The histochemical GUS assay (Section 2.8) was performed for 16 h at 22°C in darkness. Seedlings in each image are ordered from left to right: Col-8, *pNRT2.1(546)::GUS, pNRT2.1(1201)::GUS, 35S-GUS*.

3.1.3 Investigating the *pNRT2.1::LUC* reporter line

pNRT2.1::LUC seedlings were grown on vertical agar plates for 7 days, then transferred to 96-well assay plates for treatment. Seedlings were induced for 24 h with 1 mM KNO_3 and N-repressed for 24 h with 1 mM KNO_3 + 2 mM Gln. A 44-fold increase in luciferase expression was detected in the induced samples, compared to the uninduced (Fig. 3.3), whereas when 2 mM Gln was present during the NO_3^- induction the increase was only 8-fold. This indicates a very high degree of N-repression was achieved with this line under these conditions.
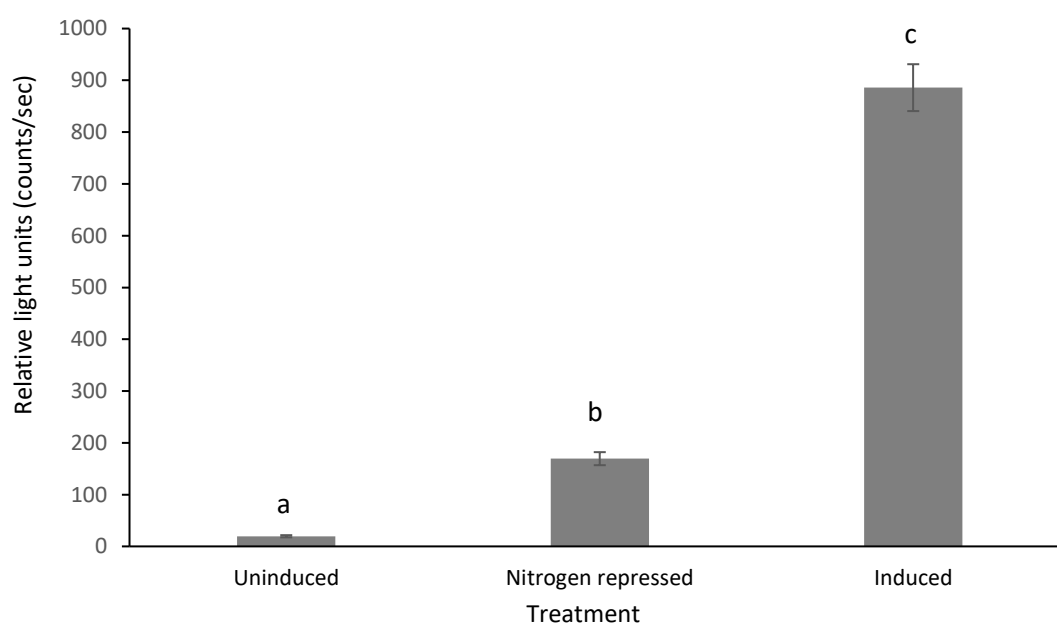
Figure 3.3 Luminescence response of *pNRT2.1::LUC* seedlings under different N regimes. Seven day old *pNRT2.1::LUC* seedlings, which had been grown on vertical agar plates containing a background level of N (50 µM Gln), were transferred to a 96- well assay plate and subjected to a 24 h N treatment: uninduced (50 µM Gln), N-repressed (1 mM KNO_3 + 2 mM Gln), Induced (1 mM KNO_3). Results are the mean of 8 (uninduced), 36 (N repressed) and 36 (induced) individual seedlings (± SE). The LUC assay contained 1 mM LUC and 4 mM ATP (Section 2.7.2) and was performed in a VictorII PerkinElmer dedicated luminometer (Section 2.7.5). Different letters denote statistically significant differences (ANOVA, $p < 0.05$, 79 degrees of freedom, LSD (5%) = 108.1).

3.1.4 Developing an 'ice capture' method to make the luciferase assay compatible with a high-throughput screening platform

Inspiration for developing a high-throughput screen came from an Arabidopsis microphenotyping platform previously described for observing changes in root architecture in a 96-well plate format (Forde et al., 2013). This platform used commercially available FrameStrips, consisting of strips of 0.3 ml PCR tubes, filled with solid nutrient medium and open at the bottom. The tubes rested in a 96-well assay plate that contained liquid nutrient medium. This allowed chemical treatments to be applied from below while root development could be observed through the clear walls of the growth tubes. The experiments described in this section were aimed at adapting this approach to allow quantitative luciferase assays to be performed on roots of the *pNRT2.1::LUC* line in a similar 96-well format, something not previously done.

The first task was to adapt the FrameStrip method to allow root tissue to be harvested from each of the growth tubes and in quantities sufficient for the luciferase assay. It was known that roots would continue to grow even after they had reached the bottoms of the FrameStrip tubes (Forde et al., 2013), raising the possibility that the protruding roots could be harvested for the luciferase assay. However, the space below the tubes in the 96-well plate was very restricted, severely limiting the amount of root material that could develop. Therefore, the FrameStrip platform was modified so that the FrameStrips could be raised by 1.6 mm once the roots had begun emerging from the tubes (after about 7 days). This was done by resting the ends of the FrameStrips on custom-made acrylic strips placed at either side of the 96-well plate. This provided an enlarged volume under the growth tubes where seedling roots could grow down into the liquid medium and accumulate (see Fig. 3.4). To accommodate the prolonged growth of seedlings in the FrameStrip method the background concentration of N was increased form 50 μM to 100 μM NH_4NO_3 and the growth medium concentration of Phytagel was reduced from 0.8% to 0.7%. By day 14 there was a sufficient mass of root material emerging from the base of the tubes to perform the luciferase assay.

The next challenge was to find a way to harvest the emerging roots separately and rapidly from each well of the assay plate. The protocol that was developed was based on the 'Ice-cap' method, which was devised as a method

for harvesting Arabidopsis root material for genotyping (Krysan, 2004). In this project, once sufficient root material had emerged from the base of the growth tubes, the FrameStrips were transferred to an assay plate containing a luciferase assay buffer. Acrylic supports were installed, raising the base of the growth tubes above the level of the buffer solution and the entire platform was left overnight at -30°C. The FrameStrips could then be broken off from the assay plate, leaving the emerging roots captured in the frozen buffer (hence the term 'ice capture'). The plate was then thawed and was ready to perform the luciferase assay.
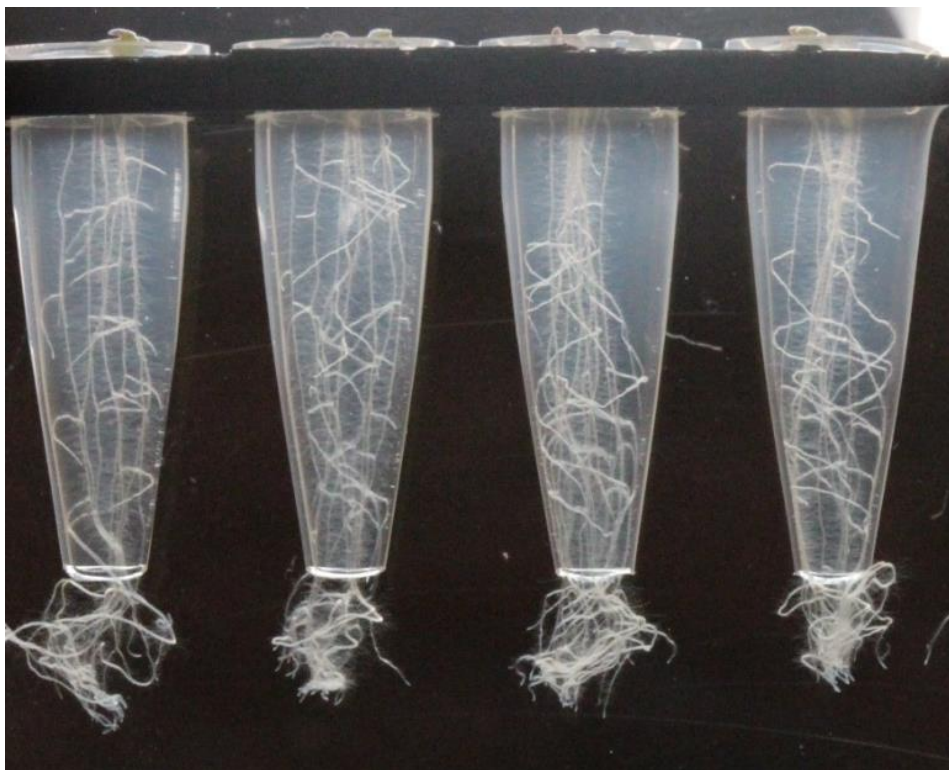
Figure 3.4 Root mass accumulation emerging from the base of the growth tubes after raising the FrameStrips The image shows how a mass of roots developed below the growth tubes that was easily accessible for harvesting samples for use in the luciferase assay.

3.2 Discussion

In order to establish the foundations of a project employing a chemical genetics approach a suitable plant reporter line needed to be identified and a screening platform designed, which was both complimentary to the plant reporter line's assay and the high-throughput principles of a chemical genetics screen. In this chapter both these criteria have been met, with the *pNRT2.1::LUC* Arabidopsis reporter line being identified and the development of the FrameStrip and 'ice capture' methods. *pNRT2.1::LUC* is suitable candidate to investigate the N signalling status of plants as the *LUC* reporter gene is under the transcriptional control of the *NRT2.1* promoter, which is under the influence of the N status of the plant. Additionally, the developed platform allows both the efficient production of root material from *pNRT2.1::LUC* seedlings which can be treated with different forms of N and efficiently harvested for a luciferase assay. In this section these achievements will discussed in further depth.

3.2.1 Identification of a suitable Arabidopsis reporter line

A screening platform must have the ability to survey thousands of bioactive compounds for an interaction with a targeted process, in a high-throughput manner. It is therefore essential that when an interaction occurs, the result is clear and definitive. The NRP-YFP reporter line displayed a clear distinction between the induced and N-repressed samples (Fig. 3.1). The intention had been to use this reporter line in conjunction with the FrameStrip method and visualise the YFP signal through the walls of the growth tubes, much like analysis of root architecture in the microphenotyping platform. However, when tested the YFP signal was undetectable through the walls of the growth tubes, even in the induced samples (data not shown). In a similar manner, had the *pNRT2.1::GUS* reporter lines been more responsive to the N-repression conditions than they proved to be (Fig. 3.2), the roots were to be stained histochemically and GUS expression was to be visualised through the sides of the growth tubes. Interestingly, a study using the *pNRT2.1(1201)::GUS* and the *pNRT2.1(546)::GUS* reporter line also could not completely repress GUS expression under high N conditions (10 mM NH_4NO_3) (Girin et al., 2007).

The results obtained with the *pNRT2.1::LUC* reporter line in this project (Fig. 3.3) were similar to the findings of Girin et al. (2010) who also demonstrated that luciferase expression was both strongly nitrate-inducible and N-repressible. However, while showing a similar repressive effect, this was achieved using 10 mM NH_4NO_3, instead of the 2 mM Gln used here (Girin et al., 2010). Our results show that despite the presence of KNO_3, Gln can strongly repress the luminescence response (Fig. 3.3), supporting the theory that end products of assimilation are major players in N-repression (Filleur et al., 2001).

Based on the very strong difference seen in luciferase activity between nitrate-induced *pNRT2.1::LUC* roots in the presence and absence of 2 mM Gln (Fig. 3.3), it was decided that this was the most suitable reporter line for the proposed chemical genetic screen. This was supported by a study showing that luciferase expression of this reporter line, analysed via enzyme assay was tightly correlated to the endogenous expression of *NRT2.1* (Girin et al., 2010).

3.2.2 Developing the FrameStrip method for high-throughput root luciferase assay analysis

Using the FrameStrip method, root growth for the first 7 days was taking place within the solid medium contained in the growth tubes. To maintain the platform's high-throughput capacity it was not feasible to access this material for the luciferase assay. Instead, bespoke supports for the Framestrips were installed to create space underneath the growth tubes that roots could grow into and accumulate in the liquid medium contained in the wells of the microtiter plate. The 'ice capture' method described in this chapter was developed to allow this root material to be harvested. The advantages of the ice capture method were that: 1. it allowed the roots growing in each well to be harvested and kept separate in the same 96-well format; 2. the rapid freezing reduced the opportunity for losing luciferase activity due to protease activity; 3. all roots on an individual plate were frozen and harvested simultaneously, which was both time-efficient and ideal for maintaining consistency across the plate. This relatively simple protocol was a key step in creating a screening platform that was compatible for use with the *pNRT2.1::LUC* reporter line.

3.2.3 Summary

In this chapter a suitable Arabidopsis reporter line (*pNRT2.1::LUC*) was identified and selected to monitor the N signalling status of the plant. *pNRT2.1::LUC*, under N- repressed conditions showed a strong reduction in luminescence (similar to the uninduced), compared to the induced. The statistically significant 8-fold change in luminescence between N-repressed and induced treatments presents an Arabidopsis line suitable for the purposes of this study. With this reporter line a novel method of culturing root material from the *pNRT2.1::LUC* seedlings was designed. This use of the FrameStrip method and bespoke 'ice capture' system designed for this research allowed root material from the *pNRT2.1::LUC* reporter line to cultured and harvested ready for analysis in a high-throughput manner. The next step was to use this novel screening platform in conjunction with *pNRT2.1::LUC* to optimise N treatment conditions, optimise the luciferase assay and statistically validate the luminescence outputs from different N treatments for the screening platform, so it may be used for the purposes of chemical genetics in this research project.

Chapter 4. Establishing the conditions for use of the *pNRT2.1::LUC* reporter line in a screen for antagonists of N-repression

In Chapter 3 it was established that of the three Arabidopsis reporter lines tested, the *pNRT2.1::LUC* line was the most suitable for the purposes of developing a chemical screen for small molecules that could interfere with N-repression of gene expression. In addition, the development of a 96-well plate system suitable for combining quantitative assays of luciferase activity in roots with the application of large numbers of small molecules was described. However, to turn this into a reliable protocol for the desired chemical genetics screen it was necessary to optimise each step in the procedure.

As described in Chapter 3, a novel 'ice capture' method was developed for frozen root material from the *pNRT2.1::LUC* line for the luciferase assay. The next objective was therefore to find the most appropriate conditions for conducting the luciferase assay using this material. Further objectives were to optimise the timing and concentrations of the reagents required to induce and repress the *pNRT2.1::LUC* gene and to identify the reduced form of N that was most effective at repression of this reporter gene.

The overall aim of this chapter was therefore to develop a protocol in which there was the clearest and most reproducible distinction between the luciferase activity measured in nitrate-induced roots and that measured in N-repressed roots. A statistical test (the *Z* factor statistical parameter) was therefore employed to establish that conditions for applying a chemical screen had been met (Halder and Kombrink, 2015).

4.1 Results
4.1.1 Optimising the luciferase assay

The conditions used as the starting point for optimising the luciferase assay were from a previous study using the *pNRT2.1::LUC* Arabidopsis reporter line (Girin et

al., 2010). The effects of varying the concentration of the substrates, luciferin and ATP, were investigated as well as the potential for using bovine serum albumin (BSA) to stabilise the luciferase activity in the roots during the freezing and thawing process.

4.1.1.1 Luciferin concentration A dose response curve for the luciferin substrate in the luciferase assay is shown in Fig. 4.1. As expected, increasing concentrations of luciferin were accompanied by an increasing amount of luminescence, up to a concentration of 0.5 mM. Above 1 mM there was no statistically significant increase in luminescence. Based on this result it was decided to use 1 mM as the standard for subsequent assays, as there was no added value in using higher concentrations.
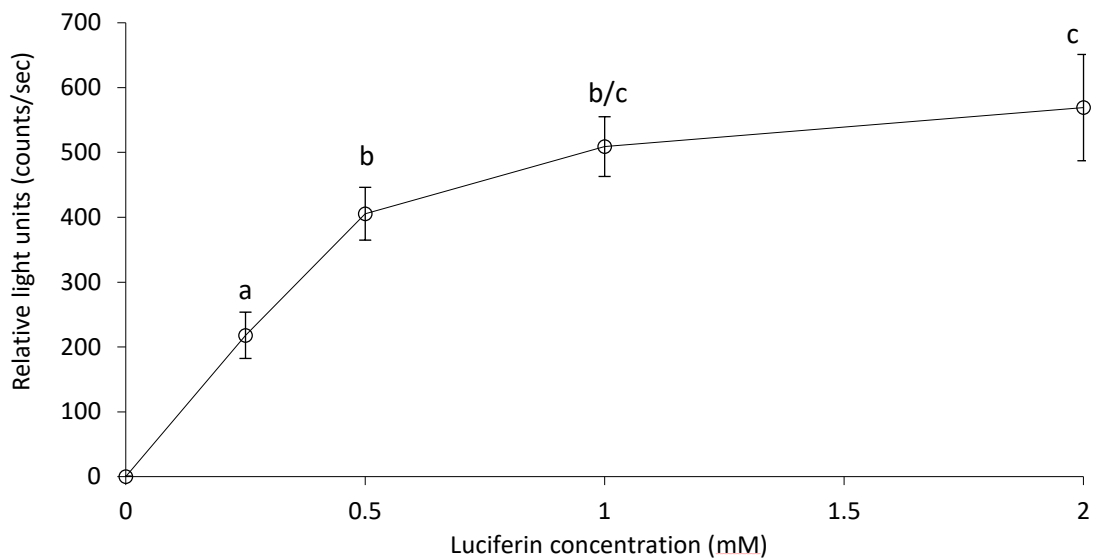
Figure 4.1 Effect on luciferase assay of varying the luciferin concentration. Seedlings of the *pNRT2.1::LUC* line (5 seedlings per tube) were grown for 14 days using FrameStrip method and then induced by treatment with 1mM KNO_3. Roots were harvested after 24 h by the ice capture method and the luciferase assay conducted on crude extracts with 5 mM ATP at a range of luciferin concentrations. Luminescence measurements were made using a Perkin Elmer Victor II dedicated luminescence microplate reader (Section 2.7.5) and background adjustments were made by subtracting mean values from wild-type samples (15 Relative light units) Values are means ± SE (n=16), Different letters denote statistically significant differences (ANOVA, $p < 0.05$, 79 degrees of freedom, LSD (5%) = 136.7).

4.1.1.2 ATP concentration A dose response curve for ATP in the luciferase assay is shown in Fig. 4.2. Increasing ATP concentrations above 1 mM produced no statistically significant increase in luminescence. Whilst there is no statically significant highest value, there is an apparent peak in mean luminescence at 4 mM ATP, which was not enhanced by increasing the concentration to 6 mM. Based on this result a concentration of 4 mM ATP was chosen as the standard for subsequent assays.
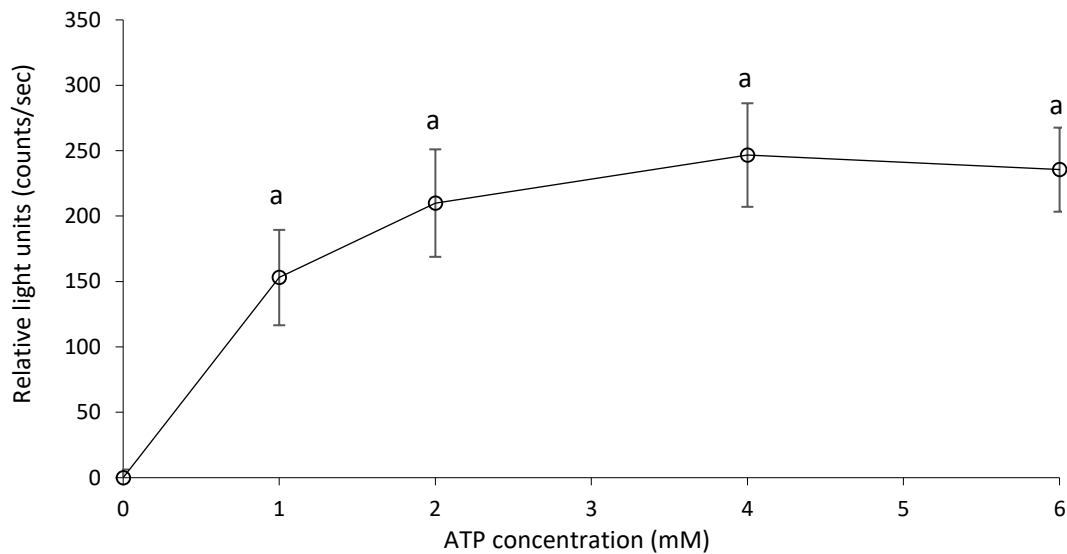
Figure 4.2 Effect on the luciferase assay of varying the ATP concentration. Root extracts were prepared as for Fig 4.1 and the luciferase assay was performed using 1 mM luciferin at a range of ATP concentrations. Luminescence measurements were made using a PerkinElmer Victor II dedicated luminescence microplate reader (Section 2.7.5) and background adjustments were made by subtracting mean values from wild-type samples (128 Relative light units). Error bars are means ± SE (n=16) and different letters denote statistically significant differences (ANOVA, $p < 0.05$, 79 degrees of freedom, LSD (5%) = 94.8).

4.1.1.3 Bovine serum albumen as a stabilising agent The process of freezing and thawing the roots in the ice capture method was an effective way to both harvest the root material and to disrupt root tissue. However, there was concern

that the freeze-thaw process might lead to denaturation of the luciferase protein and/or to its degradation by proteases released from the vacuole or other cellular compartments. Bovine serum albumen (BSA) is a well-known stabiliser of enzyme activities (Tomoyasu et al., 2013) and in sufficient quantities it could also potentially act as a sacrificial protein to mop up excess proteolytic activity in the crude root extract.

Fig. 4.3 shows that addition of BSA to the phosphate buffer in which the roots were frozen, over the range of concentrations tested (from 0 to 2 mg/ml), had no more than a minor effect on the measured luciferase activity, and statistical analysis indicated that the small positive effect at 2 mg/ml was not significant compared to the control. There was a significant difference between

0.5 and 2 mM BSA, however given the overall findings of Fig. 4.3 and what is known about BSA the reduction in luminescence at 0.5 mM BSA, which led to the significant difference when compared to 2 mM BSA was thought to be artefactual.
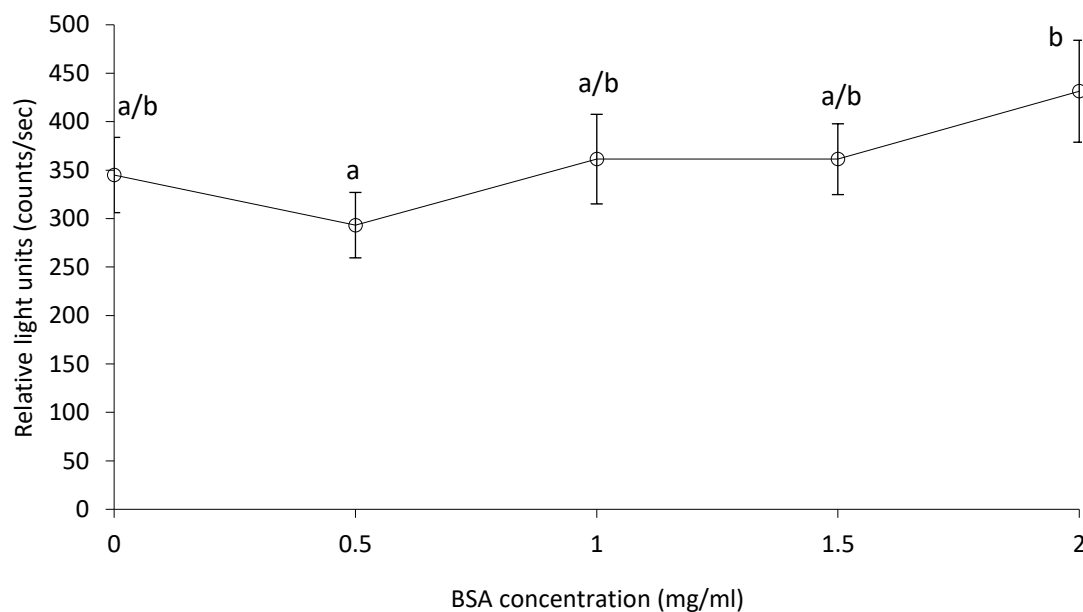
Figure 4.3 The effect on the luciferase assay of including BSA at a range of concentrations in the extraction buffer. Root extracts were prepared as for Fig. 4.1 and the luciferase assay was performed using 1 mM luciferin and 4 mM ATP in a Perkin Elmer Victor II dedicated luminescence microplate reader (Section 2.7.5). BSA was added at the indicated concentration to the phosphate buffer prior to freezing the roots. Values are means ± SE (n=16) and different letters denote statistically significant differences (ANOVA, $p<0.05$, 79 degrees of freedom, LSD (5%) = 118.8).

4.1.2 Effect of varying the concentration and duration of KNO₃ treatment on induction of *pNRT2.1::LUC* expression

Fig. 4.4 shows a time course for induction of *pNRT2.1::LUC* expression after addition of 1 mM KNO_3 to roots of 14 day old seedlings. At time zero, prior to nitrate treatment, the background level of *pNRT2.1::LUC* expression was very low. The highest level of expression was found after 24 h, the end-point of the experiment, but with most of the increase occurring during the first 12 h. An apparent decrease in activity between 3 h and 6 h was not statistically significant and was not seen in other experiments (e.g. Fig. 4.6), so is thought to be artefactual.
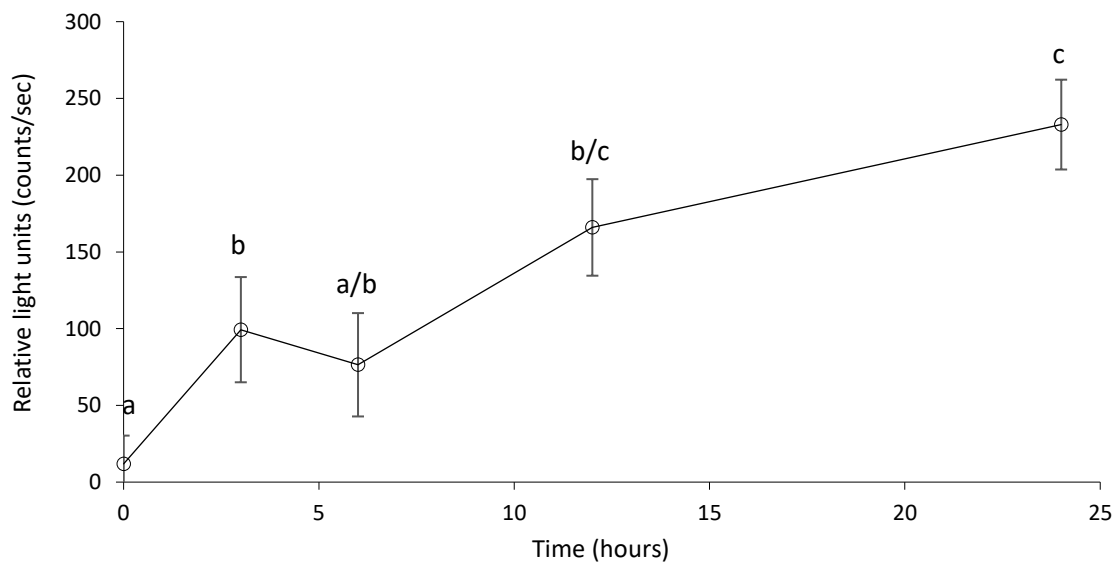
Figure 4.4 Time course of induction of *pNRT2.1::LUC* expression in roots. Seedlings of the *pNRT2.1::LUC* line were cultured for 14 days using the FrameStrip method in medium containing 50 μM NH_4NO_3. At Time 0, 1 mM KNO3 was added to the solution in which the roots were bathed and roots were frozen at intervals up to 24 h after the start of the treatment. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a Perkin Elmer Victor II dedicated luminescence microplate reader (Section 2.7.5). Values are means ± S.E. (n = 16) and different letters denote statistically significant differences (ANOVA, $p < 0.05$, 75 degrees of freedom, LSD (5%) = 84.5)**.**

Based on these results, a second experiment was performed to investigate the effect of varying the nitrate concentration on luciferase activity at two time points, 3 h and 24 h. As shown in Fig. 4.5, maximum luciferase expression was seen after 24 h in 0.3 mM KNO_3, with higher concentrations (1 mM and 5 mM) producing slightly lower expression levels at this time point. At the earlier time point (3 h), the activities were much more variable, and were consistently much lower than at 24 h at all concentrations. In this case the highest values were seen at the highest nitrate concentration (5 mM).
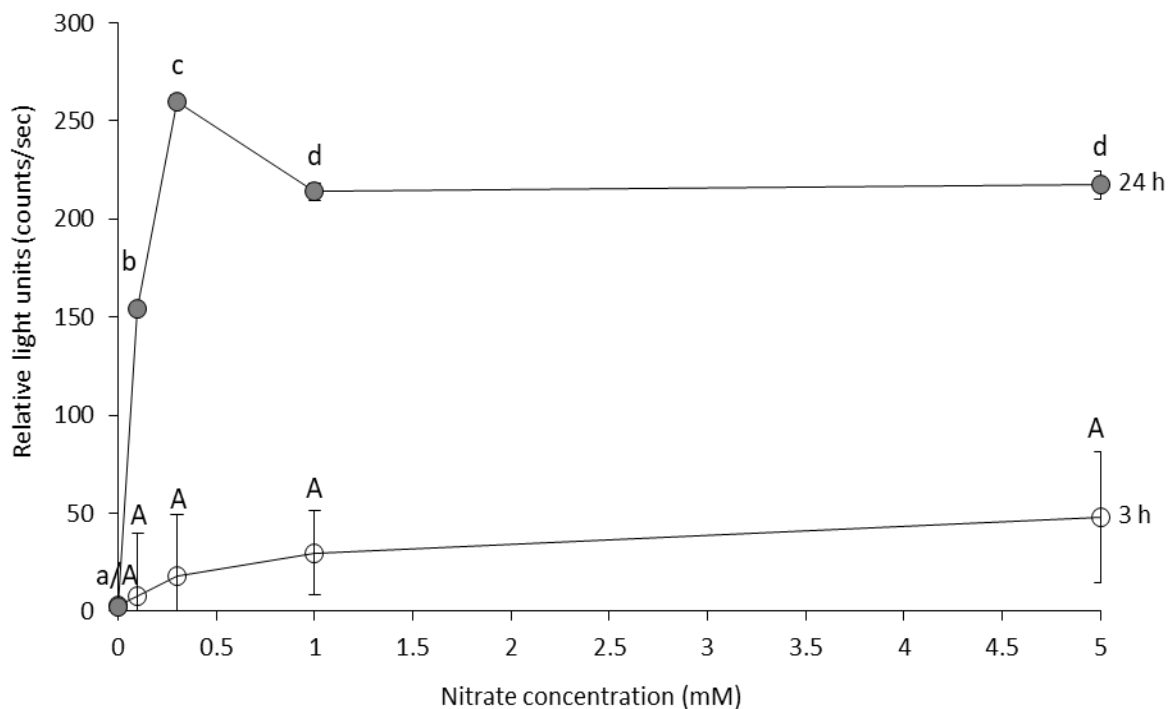
Figure 4.5 Effect of a range of concentrations of KNO3 on *pNRT2.1::LUC* expression at two time points after induction. Seedlings of the *pNRT2.1::LUC* line were cultured for 14 days using the FrameStrip method in medium containing 50 µM NH_4NO_3. At Time 0, KNO_3 (at 0, 0.1, 0.3, 1 or 5 mM) was added to the solution in which the roots were bathed and roots were frozen at either 3 h (open circles) or 24 h (closed circles) after the start of the treatment. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a Perkin Elmer Victor II dedicated luminescence microplate reader (Section 2.7.5). Values are means ± SE (n = 16) and different letters denote statistically significant differences (ANOVA, $p < 0.05$, 152 degrees of freedom, LSD (5%) = 37.29).

Based on these results it was decided to select the two concentrations of KNO₃ that gave the highest luciferase activities at 3 h and 24 h, respectively and to perform a more detailed time course with each. The results in Fig. 4.6 show that over the 24 h period after the start of treatment with 0.3 mM KNO₃, there was a linear increase in luciferase activity. While the 5 mM treatment produced a much stronger linear increase in luciferase activity (a rate of increase that was approximately twice that seen at 0.3 mM), this peaked at 12 h, after which the activity had declined significantly by the 24 h time point. Thus, the maximum activity obtained with the two concentrations of nitrate was similar, but in the case of the 0.3 mM treatment this was seen at 24 h, while in the case of the 5 mM treatment it was seen at 12 h. In subsequent experiments 0.3 mM KNO₃ and 24 h was used for induction on the basis that the positive effects of the lower
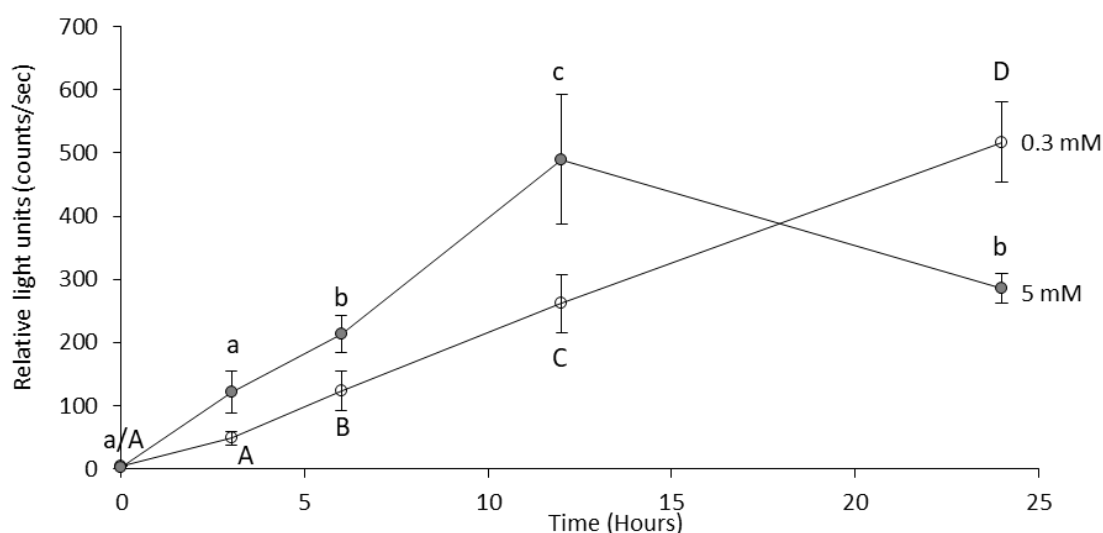
Figure 4.6 Comparison of the effects of two different concentrations of KNO₃ on the time course of induction of *pNRT2.1::LUC* expression. Seedlings of the *pNRT2.1::LUC* line were cultured for 14 days using the FrameStrip method in medium containing 50 µM NH₄NO₃. At Time 0, KNO₃ (at 0.3 mM (open circles) or 5 mM (closed circles)) was added to the solution in which the roots were bathed and roots were frozen at intervals up to 24 h after the start of the treatment. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values are means ± SE (n = 16) and different letters denote statistically significant differences (ANOVA, p<0.05, 142 degrees of freedom, LSD (5%) = 94.6).

concentration of nitrate would be uncomplicated by the negative effects seen after 24 h with 5 mM KNO$_3$.

4.1.3 Investigating the effect of the timing and concentration of Gln treatment on repression of nitrate-induced *pNRT2.1::LUC* expression

A Gln treatment was initially chosen as the means to generate N-repression, based on previous evidence of Gln's effectiveness as a N source for plants and its ability to down-regulate expression of *NRT2.1* (Nazoa et al., 2003, Zhuo et al., 1999). For the purposes of the chemical screen it was important that any repressive effect was sufficiently strong to produce levels of expression that were clearly distinguishable from those seen in the absence of Gln (nitrate alone). It was therefore decided to investigate the concentration of Gln necessary to achieve the highest level of N-repression and to determine whether the strength of the effect could be increased by applying the Gln in advance of the nitrate treatment. The idea of a Gln pre-treatment was that it would allow additional time for the Gln to enhance the N status of the plant and thereby strengthen its repressive effect.

The results presented in Fig. 4.7 show that applying Gln at a concentration of 0.2 mM simultaneously $_{a/b}$ı the inductive nitrate treatment had a strong repressive effect on *pNRT2.1::LUC* expression. At 2 mM Gln, luciferase activity in root extracts was slightly lower than at 0.2 mM Gln and approximately one third of that seen in the absence of Gln. Applying Gln 3 h before ni ^{B} ə induction led to no significant increase in its repressive effect at any of the concentrations tested. Furthermore, supplying Gln 72 h in advance greatly diminished its repressive effect, such that significant repression was only seen at the highest Gln concentration (2 mM).
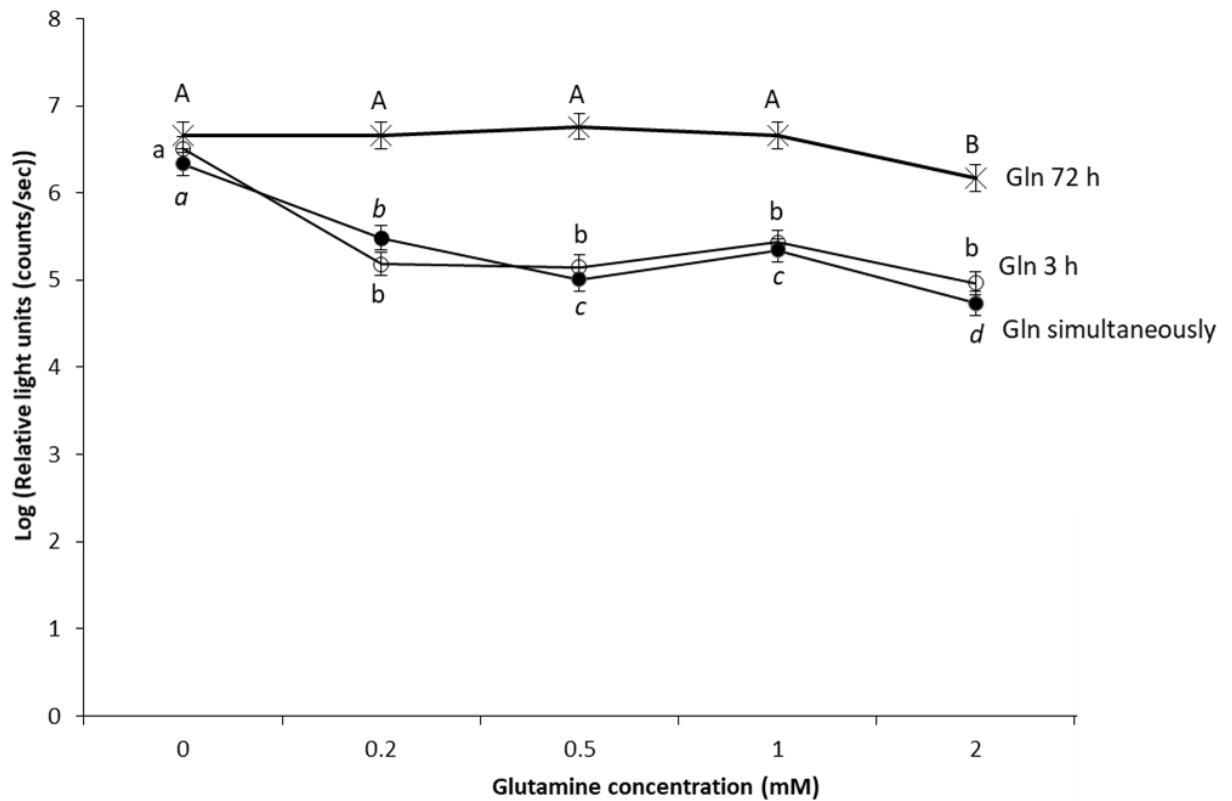
Figure 4.7 Investigating the effect of the timing and concentration of Gln treatment on repression of nitrate-induced *pNRT2.1::LUC* expression. Seedlings of the *pNRT2.1::LUC* line were cultured using the FrameStrip method in medium containing 50 µM NH_4NO_3 and nitrate induction was performed on 14 day old seedlings using 0.3 mM KNO_3. Gln treatments were applied at a range of concentrations either at the same time as the nitrate treatment (closed squares), 3 h in advance of the nitrate treatment (closed circles) or 72 h in advance of the nitrate treatment (open circles). Roots were harvested and frozen 24 h after the start of the nitrate treatments. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values are log transformed means ± LSD (n = 16) and different letters denote statistically significant differences (ANOVA, $p < 0.05$, 145 degrees of freedom, LSD (5%) = 0.14).

4.1.4 Comparing the effectiveness of a range of different amino acids in repressing nitrate-induced *pNRT2.1::LUC* expression.

As Gln, even at the highest concentration tested, did not fully repress *pNRT2.1::LUC* expression (Fig. 4.7), it was decided to investigate whether stronger effects could be achieved with other proteinogenic amino acids. Based on the results of the previous experiment, each of the 19 amino acids (including Gln) were applied to seedlings simultaneously with the nitrate treatment at two concentrations, corresponding to 0.4 mM N and 4 mM N, and luciferase activity was assayed after 24 h.

The results presented in Fig. 4.8 show that there was considerable variation in the degree to which different amino acids affected *pNRT2.1::LUC* expression. Two amino acids (Arg and His) had no significant repressive effect, even at 4 mM compared to the nitrate treatment alone. Indeed, Arg treatment had a strong stimulatory effect on expression of the reporter gene that was greater at 4mM than at 0.4 mM: at 4 mM Arg there was a 3-fold increase in *pNRT2.1::LUC* expression compared to the nitrate treatment alone. A large group of amino acids (Ala, Asn, Asp, Cys, Glu, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp and Val) produced effects that were similar, not significantly different to those seen with Gln (i.e. reducing luciferase activity by 3-fold when compared to the nitrate treatment alone). The only amino acids that produced a repressive effect that was markedly stronger than that produced by 2 mM Gln were Cys (at 4 mM), Glu (at 4 mM), Thr (at 4 mM) and Val (at 4 mM).
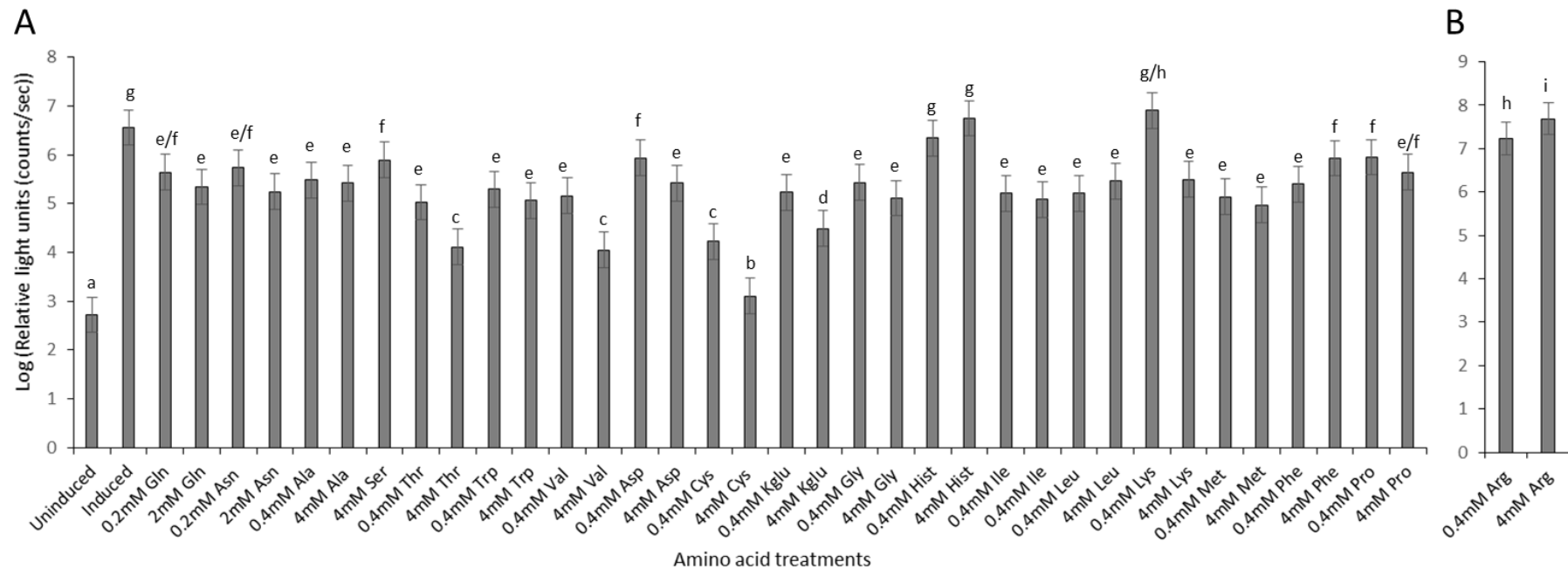
Figure 4.8 Comparison of the effectiveness of 19 different proteinogenic amino acids at repressing expression of nitrate-induced *pNRT2.1::LUC.* Seedlings of the *pNRT2.1::LUC* line were cultured using the FrameStrip method in medium containing 100 µM NH_4NO_3 and nitrate induction was performed on 13 day old seedlings using 0.3 mM KNO_3. Individual amino acid treatments were applied simultaneously with the nitrate treatment for all amino acids (A) except Arg (B) and root material was frozen after 24 h. Luciferase activity was measured in root extracts using 4 mM ATP and 1mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values are log transformed means ± LSD (n = 8) and different letters denote statistically significant differences (ANOVA, $p < 0.05$, 281 degrees of freedom, LSD (5%) = 0.37).

In a follow-up experiment, the response to selected amino acids was investigated in more detail, in particular the unexpected stimulation of *pNRT2.1::LUC* expression by Arg. Two questions were addressed: would Arg on its own, in the absence of nitrate, stimulate *pNRT2.1::LUC* expression and would the presence of Arg block the ability of other amino acids to repress *pNRT2.1::LUC* expression? As shown in Fig. 4.9, neither Arg on its own, nor any other amino acid tested (Gln, Met and Cys) had any significant effect on *pNRT2.1::LUC* expression in the absence of nitrate. In this experiment Arg at both 4 mM and 8 mM stimulated *pNRT2.1::LUC* expression approximately 2-fold in the presence of nitrate, with 0.4 mM having a slightly smaller, yet still statistically significant effect. When other amino acids were included alongside Arg it was found that the ability of Cys and Met to repress *pNRT2.1::LUC* expression (compared to the nitrate + Arg treatments) was as strong as in the absence of Arg. The same observation was made for Gln in the presence of 0.4 mM Arg, while treatments in the presence of 4 mM and 8 mM Arg were slightly less effective, but still significantly under that of the nitrate + Arg treatment. Thus the stimulatory effect of Arg in the presence of nitrate does not appear to be due to any interference with the normal processes that lead to N-repression of *pNRT2.1::LUC* expression as in all cases the signal could be significantly repressed.
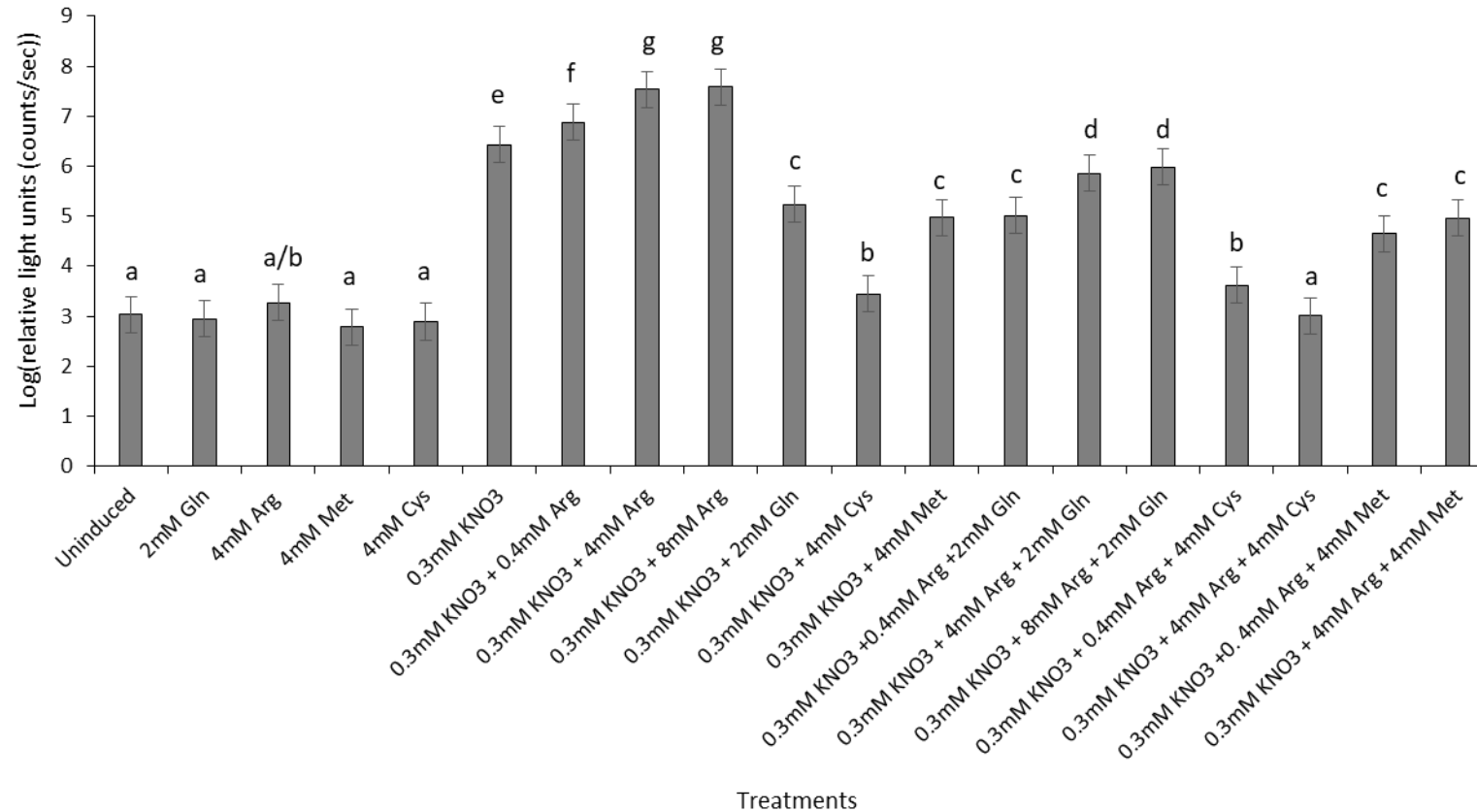
Figure 4.9 Investigating the stimulatory effect of Arg on *pNRT2.1::LUC* expression. Treatments were performed on 14 day old seedlings of the *pNRT2.1::LUC* line as for Fig. 4.8. Treatments with nitrate or amino acids alone, or combinations of nitrate and one or more amino acids as indicated in the Fig. 4.8, were applied simultaneously and roots frozen 24 h later. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values are log transformed means ± LSD (n = 8) and different letters denote statistically significant differences (ANOVA, p<0.05, 149 degrees of freedom, LSD (5%) = 0.36).

4.1.5 Applying the *Z* factor test

The *Z* factor test is a statistical method that was developed to assess the quality of an assay to be used in a high-throughput screen, as a more rigorous alternative to simply looking at the signal to noise ratio (Zhang et al. 2009). It is a method for assessing how reliable the differences are between the positive controls and the negative controls. Negative values indicate an unacceptable assay, while positive values are acceptable (with a value of 1.0 being the theoretical maximum).

In a preliminary experiment with the *pNRT2.1::LUC* line, 96-well plates were set up in which the luciferase activities in eight nitrate-induced positive control samples (and eight negative control samples, containing background N only) were compared with 80 nitrate-induced samples treated with 2 mM Gln (N-repressed; Fig. 4.10A). The value obtained for the *Z* factor for this experiment was -0.20, indicating an unacceptable assay. To try to improve the quantitative difference between the induced and N-repressed samples, the experiment was repeated using 10 mM Gln (Fig. 4.10B) and a *Z* factor of 0.04 was obtained, indicating that the assay was just acceptable. In an attempt to improve the quality of the assay further, an additional amino acid in the form of Arg was included alongside Gln (2 mM Gln + 4 mM Arg) and 4 mM Arg was also included alongside the nitrate in the induction treatment, on the basis that Arg was previously found to have a positive effect on nitrate induction (as seen in Fig. 4.8 and 4.9). However, this experiment produced a *Z* factor of -0.29 (Fig. 4.10C). In a fourth experiment an alternative high N-repression treatment was investigated (10 mM NH_4NO_3) (Fig. 4.10D). However, there was no improvement on the previous conditions, producing the lowest *Z* factor of -0.92. In a fifth experiment the concentration of amino acids in the N-repression treatments was increased further (10 mM Gln + 4 mM Arg) and 4 mM Arg was again included in the nitrate induction treatment (Fig. 4.10E). In this case the *Z* factor was increased to 0.27, indicating an improved level of acceptability for the assay.
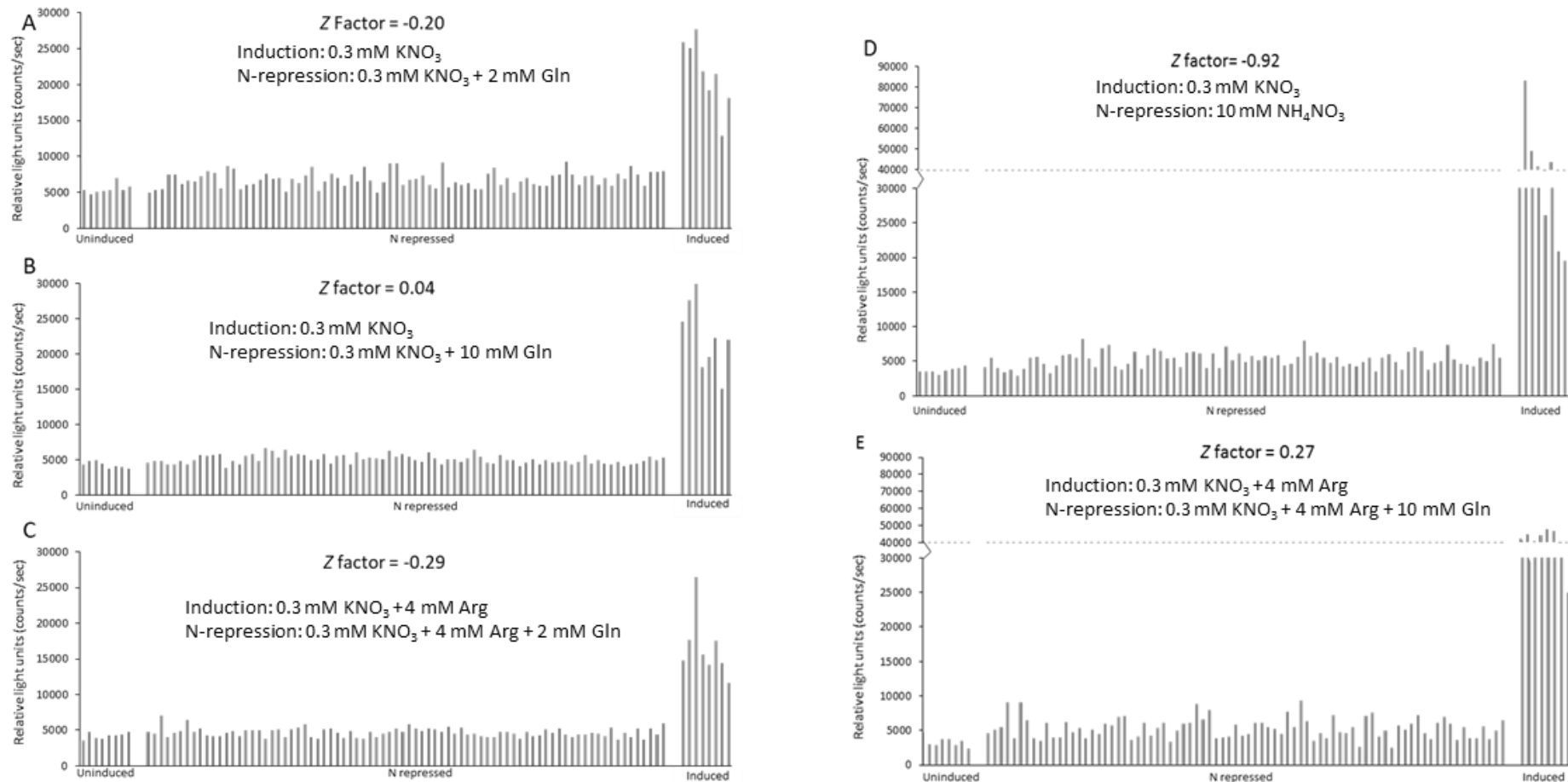
Figure 4.10. Optimising treatments to obtain acceptable *Z* factor values for the N-repression screen. Treatments were performed on 14 day old seedlings of the *pNRT2.1::LUC* line in 96-well plates as for Fig. 4.8 and root samples were frozen after 24 h. Luciferase assays were performed on root extracts in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6) and *Z* factors calculated as described in Section 2.11.2.

4.2 Discussion

Building on the preliminary experiments reported in the previous chapter, Chapter 4 presents experimental data which optimised each step of the protocol required for the chemical genetics approach. The aim of this optimisation was to produce a reliable protocol which would allow the most suitable conditions for the luciferase assay and N treatment conditions to be established. Optimisation of the luciferase assay reagent composition and concentration was performed over different time periods to produce the most suitable conditions for the assay. The optimised luciferase assay was then used to investigate the most suitable induction and repression N treatment conditions. After optimising N treatment conditions, including an investigation of the potential for 19 different amino acids at two concentrations to repress *NRT2.1* expression in the presence of nitrate the platform was validated using the *Z* factor statistical parameter. In this section these achievements will be further discussed.

4.2.1 Optimisation of the luciferase assay

Firefly luciferase, produced by *pNRT2.1::LUC* catalyses an oxidative reaction between luciferin, ATP and molecular oxygen to create an excited oxyluciferin species (Baldwin, 1996). This species produces photons that are measured as luminescence. If the luciferin substrate or ATP are insufficient in concentration the reaction is limited. The concentrations required for any luciferase assay using a plant luciferase reporter line are dependent on the amount of biological tissue present and the effectiveness of the luciferase enzyme extraction from that tissue, hence the importance for optimisation in this project. Here we established that, at 1 mM luciferin and 4 mM ATP these reagents were near saturating the luciferase assay, therefore neither were a limiting factor. The assay did not contain a detergent, it relied on the freeze-thaw 'ice capture' method to lyse cells for luciferase enzyme release (preliminary experiments without the freeze-thaw step produced undetectable levels of luciferase expression, data not shown). Detergents, as hydrophobic substances are known to non-specifically bind to the luciferase enzyme, stimulating catalytic activity and interfering with luciferase enzyme detection (Lembert and Idahl, 1995, Kricka and Deluca, 1982). BSA is

also known to stimulate light promotion (Kricka and Deluca, 1982), however given that the freeze-thaw cycle could have damaged the intracellular luciferase enzyme its properties as a cryoprotectant were investigated. As there was no statistical benefit observed from the luminescence output of luciferase assays containing BSA it was omitted from further testing.

High combinations of luciferin and ATP have been shown to produce a strong initial burst of light followed by a state of inactivity (Lembert and Idahl, 1995). To account for this, the luciferase assays described in this chapter were performed in a luminometer with a reagent injection system (LUMIstar Omega, BMG LABTECH). The injection system allowed delivery of the luciferin, mixing and detection to occur immediately so this initial burst which occurs within a matter of seconds could be measured. An alternative method would have been be to pre-incubate the luciferin with the crude root samples prior to 'ice capture' freezing, which has been shown to stabilise luminescence output (Lembert and Idahl, 1995). Alternatively, the use of coenzyme-A, acting as a substrate has been shown to reduce luminescence half-life (Fraga et al., 2005). However, once the luminometer with a reagent injection system became available it was not necessary to pursue these alternative strategies.

4.2.2 Optimisation of nitrate induction

In this chapter 0.3 mM KNO_3 has been shown to produce the strongest induction of *pNRT2.1::LUC* luminescence over a 24 h period (Figs. 4.5 and 4.6). Girin et al. (2010) used the same nitrate concentration to produce a similarly strong luciferase induction in this line, with induction closely correlated to the *NRT2.1* transcript. In an earlier publication, Girin et al. (2007) used the same promoter sequence for a GUS transgene, where induction of GUS expression was highest between 0.3-0.5 mM KNO_3. In agreement with Fig. 4.5 (24 h treatment), their findings show GUS expression was positively correlated to increases in KNO_3, at low concentrations (0-0.5 mM) and repressed at higher concentrations (5-20 mM KNO_3) (Girin et al., 2007). The previous studies differed from the present research by seedlings being grown at the treatment concentration of KNO_3 for six to seven days (from the start of germination), rather than applying a short-term (24 h) treatment to N-depleted seedlings. Zhou et al. (1999) found that when

Arabidopsis seedlings were grown on 1 mM ammonium citrate, before being transferred to 1 mM KNO_3, the induction peak was reached after only 3 h incubation. This differs from the results shown in Fig 4.5 where induction after 24 h was much stronger than after 3 h at all KNO_3 concentrations tested (0.1 – 5 mM). It seems likely that the explanation for the difference lies in the higher N status of the ammonium citrate-grown seedlings which could lead to more rapid feedback repression of the *NRT2.1* gene. Nazoa et al. (2003), working with the *pNRT2.1(1201)::GUS* line, imposed a 3 h N-free period before performing a time course assay with 2 mM KNO_3. Increasing the time of exposure to nitrate, up to 24 h produced increasingly higher levels of GUS expression, similar to the results of Fig. 4.4. In agreement with Fig. 4.6 they showed that lower nitrate concentrations (0.2 mM) stimulated *NRT2.1*- dependent reporter activity, while higher concentrations (2 - 10 mM) repressed the signal.

Studies on *NRT2.1* nitrate response in other higher plants show similar findings. Amarasinghe et al. (1998) report that *NRT2.1* transcript levels in soybean were most strongly increased when plants were N-starved prior to a 1 mM KNO_3 application, compared to being continually grown at 1 mM KNO_3. Additionally, they found that incubation with nitrate up to 6 h resulted in an accumulation of the *NRT2.1* transcript. However it was repressed at 24 h (Amarasinghe et al., 1998), similar to the 5 mM KNO_3 treatment in Fig. 4.6. Such a trend was also shown in barley, where low concentrations of nitrate (1 mM KNO_3) increased *NRT2.1* expression up to 9 h, after which transcript levels declined, with higher concentrations (10 mM). It would seem that increasing the concentration of nitrate results in an increased rate at which peak luminescence is reached, followed by the subsequent decline as expression is repressed (Vidmar et al., 2000b). This effect, seen in Fig. 4.6 is further supported by studies on spring wheat, where a low nitrate concentration (0.2 mM) does not produce this repression over 24 h (Zhao et al., 2004).

The discussed literature, in conjunction with Figs. 4.4 – 4.6 show that the rate at which induction of *NRT2.1* is achieved is clearly dependent on the KNO_3 concentration and incubation time. Low and high KNO_3 concentrations cause induction of *NRT2*.1, with higher concentrations producing peak induction earlier than lower concentrations. In turn, higher concentrations then result in earlier and stronger N-repression of *NRT2.1*. In agreement, Filleur and Daniel (1999)

demonstrated a similar *NRT2.1* expression pattern in response to a low and high concentration of KNO_3, using 50 μM and 50 mM. Additionally, the background N concentration is influential, with *NRT2.1* being more responsive to KNO_3 with a N-free pre-treatment. Hence the reason that the background N concentration in the period before nitrate induction was kept low (50 μM NH_4NO_3), to ensure the seedlings were depleted of N before induction was initiated.

4.2.3 Optimisation of the timing and concentration of Gln treatment

High nitrate concentrations repress *NRT2.1* expression (Girin et al., 2007, Girin et al., 2010, Nazoa et al., 2003), however the mechanism for this is still unclear. Previous research suggests that there are several interactions that occur to elicit N-repression. A well supported theory is that reduced forms of N play a major role in repressing *NRT2.1* expression. The strong negative effect of Gln was demonstrated, repressing *NRT2.1* in the presence of inductive concentrations of nitrate. In agreement, Nazoa et al. (2003), using the *pNRT2.1::GUS* Arabidopsis line showed that Gln (10 mM) had a strong repressive effect on GUS expression, stronger than ammonium nitrate. They also reported that the endogenous application of ammonium or amino acids (Gln, Ala, Arg, Asp, Asn) reduced *NRT2.1* expression. Interestingly, it was observed that regardless of the amino acid applied, internal concentrations of Gln increased, consistent with the notion that Gln is one of the main elicitors of N-repression (Nazoa et al., 2003). Zhou et al. (1999) reported that the application of 1 mM KNO_3 with inhibitors Aminooxyacetate (AOA) and Azaserine (AZA), resulting in a higher internal Gln and Glu concentration, displayed a reduced *NRT2.1* transcript. Interestingly, using 1 mM Gln application they did not see a repression of the *NRT2.1* transcript which conflicted with their own data on Gln-repression (using AOA and AZA) and Fig. 4.7 in this research.

Figure 4.7 shows that the exogenous application of Gln (2 mM) represses *NRT2.1* expression. Interestingly, a 3 h pre-treatment with Gln did not enhance *NRT2.1* repression, when compared to its simultaneous application with KNO_3. This unexpected result suggests that at the time of root harvest both treatment groups had a similar N status. One explanation could be that Gln altered the N status of the plants to its maximum extent for the specific N concentration within

24 h, therefore regardless of pre-treatment the results would be the same. Another possible, somewhat controversial explanation could be that the current external Gln conditions or the fluctuations of Gln through the plants, rather than its N status were more influential on the expression of *NRT2.1*.

Interestingly, a 72 h pre-treatment only had a significant effect at the highest concentration of Gln (5 mM), lower concentrations had a greatly diminished effected compared to the 3 h pre-treatment and simultaneous application (Fig. 4.7). It is most likely that external Gln at <5 mM was depleted before KNO_3 induction, which would happen more quickly at lower concentrations, hence why only 5 mM Gln produced an effect. In relation to the theory proposed to support the 3 h pre-treatment results, if Gln was depleted it would support the idea that the external concentration of Gln or its fluctuation through the plant was more influential than the N status itself. This being the case as it was expected that seedlings, exposed to those Gln treatments would have had sufficient time to develop an improved N status. This raises questions about what is meant by the N status of plants and what mechanisms are most important in triggering N-repression. To help answer this question the experiment in Fig. 4.7 could be repeated, but with analysis of different N source concentrations in the external conditions, rate of N uptake, plant internal amino acid pool compositions and expression of *NRT2.1.*

Other research using the *pNRT2.1::LUC* and *GUS* reporter lines showed that alternative sources of high N (10 mM NH_4NO_3) were effective in repressing *NRT2*.1 (Girin et al., 2010, Girin et al., 2007). Experimentation with the GUS line showed that the application of NH_4NO_3 was much more effective at repressing *NRT2.1*- dependent GUS expression than an equal concentration of Gln (Girin et al., 2007). Zhuo et al. (1999) reported that separate KNO_3 and NH_4NO_3 treatments used to induce *NRT2.1* were strongly repressed in the presence of L-methionine sulphoximine, which inhibits the conversion of NH_4^+ to Gln. These results suggest that NH_4^+ also plays a strong role in *NRT2.1* repression.

4.2.4 Effectiveness of other amino acids at exerting N-repression

As expected, the application of amino acids in the presence of nitrate represses *NRT2.1* expression in general, compared to the nitrate induced. As shown in Fig.

4.8A most amino acids are as effective at repressing *NRT2.1* as Gln, however most notably 4 mM Cys was significantly more powerful. Interestingly we observed that His had little to no effect, while the higher concentration of Lys promoted *NRT2.1* expression. Nazoa et al. (2003) in agreement with Fig. 4.8A reported that Gln, NH_4^+, Ala and Glu all had inhibitory effects on *NRT2.1* expression. The result from these studies, an application of reduced forms of N resulting in the down-regulation of *NRT2.1* was expected, particularly for Asn, Asp, Gln and Glu as they are involved in the initial steps of the N assimilation pathway. Interestingly, Nazoa et al. (2003) also reported that of the amino acids tested, all resulted in an increase in internal Gln concentrations within the plants. Other research similarly reported that NH_4^+, NH_4NO_3, Asn and Gln repress *NRT2.1* in Arabidopsis (Girin et al., 2007, Zhuo et al., 1999). A study on barley also showed that in the presence of KNO_3, Asn and Gln repressed *NRT2.1*, as well as Asp and Glu which had a significantly stronger effect (Vidmar et al., 2000b), similar to the findings of Fig. 4.8A. Together these findings suggest that reduced forms of N, particularly Gln have a major negative influence on *NRT2.1* expression.

Most counter-intuitively, it was found that Arg strongly promoted *pNRT2.1::LUC* expression, increasing the luminescence signal by up to 3.2-fold (Fig. 4.8B). This contrasts with the results of a previous study by Zhuo et al. (1999) in which it was found that that Arg strongly repressed *NRT2.1* expression. There were a number of differences between the earlier study and the present one that might account for this discrepancy. Firstly, they were studying the expression of the endogenous *NRT2.1* gene, so that they could have detected effects that required sequences within the transcribed region of the gene or that acted at the post-transcriptional level. Secondly, there were significant differences in the culture conditions between the two studies, notably their use of hydroponics and their choice to pre-culture the seedlings in a relatively high concentration of ammonium succinate (Zhuo et al., 1999). The latter differences in growth conditions might also account for why they also found that Gln did not repress nitrate induction of *NRT2.1*. A comparison of the setup reveals that they observed gene expression after a 3 h treatment period, which we reported to have a much reduced effect on nitrate induction compared to a 24 h treatment (Fig. 4.5). While Zhuo et al. (1999) were able to demonstrate a strong nitrate

induction in 3 h, they acknowledge that the results, using L-methionine sulfoximine and aminooxyacetate with azaserine to increase amino acid content contradict the lack of repression reported for Gln which contradicts not only the findings here, but also those of most other published studies (Girin et al., 2007, Nazoa et al., 2003, Vidmar et al., 2000b).

4.2.5 How does Arg stimulate expression of *pNRT2.1::LUC*

An initial hypothesis from Fig. 4.8 was that Arg itself was stimulating expression of *pNRT2.1::LUC,* independently of KNO_3. It is known that a plant pathway for the biosynthesis of nitric oxide (NO), an important signalling molecule (Gupta et al., 2011, Domingos et al., 2015, Thalineau et al., 2016) uses Arg as a substrate. In the N assimilation pathway the reduction of nitrate and nitrite produces NO as a by-product (Gupta et al., 2011). It is possible that the supply of Arg, produced by the discussed methods promoted an accumulation of NO, which somehow stimulated *pNRT2.1::LUC* expression. Previous studies with green algae, *Chlamydomonas reinhardtii*, reported that NO inhibited the high-affinity uptake of nitrate, nitrite and ammonium as well as reducing nitrate reductase activity (Sanz-Luque et al., 2013). This inhibition could suggest that in this research NO, reducing the further uptake of N into the roots and inhibiting N assimilation allows the already imported nitrate to persist for longer in the plant with a lower accumulation of reduced forms of N. This combination could be responsible for the stimulated expression of *pNRT2.1::LUC*, as the persisting nitrate stimulates *NRT2.1* in the absence of repression exerted by accumulated amino acid pools.

To test the hypothesis that Arg-dependent NO signalling might be responsible for super-induction of *pNRT2.1::LUC*, independently of nitrate, the effect of Arg on *pNRT2.1*::*LUC* expression in the absence of nitrate was tested (Fig. 4.9). However, it was found that Arg on its own had no significant effect on the expression of the *pNRT2.1::LUC* construct and that to see a stimulation a low concentration of KNO_3 was additionally required. A new hypothesis could be that the presence of Arg, by stimulating NO production has an indirect nitrate-dependent effect on *pNRT2.1::LUC* expression by inhibiting NR activity and thereby causing imported nitrate in the roots to persist at higher concentrations for longer. This could be tested by applying an inhibitor of NR, such tungstate and

then analysing the endogenous gene expression of *NRT2.1*, *NIA1, NIA2* and *NIR*. A previous study using an NR-deficient G' 4-3 Arabidopsis mutant showed that in this plant reporter line *NRT2.1* induction was nitrate-induced nearly 2.5-fold more strongly than the wild-type (Filleur and Daniel-Vedele, 1999). Thus, any treatment that inhibits NR activity would be expected to have the same effect.

4.2.6 Assessment of the robustness of the screen for antagonists of N-repression

The *Z* factor statistical parameter (Zhang et al., 1999) was designed for the evaluation of high-throughput screening assays. Specifically it judges the signal's dynamic range and measurement variation, allowing the assay to be quantatively optimised and validated. In this research it was used to judge whether the luminescence output of the two treatment groups (N-repressed and Induced) was large enough to be statistically difference, while accounting for the variation within each group. By satisfying the *Z* factor statistical parameter it demontrates: 1) Each treatment groups' luminescence output is consistent and sufficiently different from the other group to allow the clear identification of an antagonist of N-repression when screening chemical libraries. 2) The variation within and the difference between each treatment group does not mask or exacerbate the effect of chemicals applied whilst screening the chemical libraries.

The use of 2 mM Gln to N repress the luminescence signal (Fig. 4.10A) returned a *Z* factor value that was too low (*Z* = -0.2), indicating that there was not a sufficient difference between the N-repressed group and the induced group. By increasing the concentration to 10 mM Gln (Fig. 4.10B) the N-repressed sample group had a reduced average luminescence, along with less variation allowing the assay to be sucessfully validated (*Z* = 0.04). Attempting to improve the statistical validation of the assay other conditions for N-repression were investigated. In one experiment, 10 mM NH_4NO_3 was used to N-repress samples, as other studies using the *pNRT2.1::LUC* and *pNRT2.1::GUS* lines had reported a strong effect (Girin et al., 2007, Girin et al., 2010). However, using 10 mM NH_4NO_3 returned the Z factor to an unsatisfactory value (*Z*= -0.92) (Fig. 4.10D), being less suitable as a means to N-repress the samples than 2 mM Gln. Nazoa

et al. (2003) similarly found that Gln was more effective at repressing *NRT2.1* than NH_4NO_3 when both were used at 10 mM.

In an attempt to improve the *Z*-factor further, Arg was included in two further trials, based on its ability to super-induce *pNRT2.1::LUC* and for Gln to still be able to repress this signal. Arg was included with both the low and high concentration of Gln (2mM and 10mM) in separate plates. The plate containing 10 mM Gln for N repression also had 4 mM Arg to stimulate N induction and an improved *Z* value of 0.27 was obtained (Fig. 4.10E). However, the plate that contained 4 mM Arg and 2 mM Gln (Fig. 4.10 C) returned an unsatisfactory value (*Z*= -0.29).

Validation of the screening platform using 4 mM Arg and 10 mM Gln with the *Z* factor statistical parameter showed that the screening platform was statically viable and functionally suitbale for the purposes of screening chemical libraries in the search for anatagonists of N-repression. The *Z* factor statistical parameter, unlike other statical tools was able to confirm that under the specified conditions, each data point within a treatment groups was suitably similar, yet significantly different than those in the other treatment group (i.e. N-repressed and Induced).

Considering the preliminary experiments that informed the N treatment regimes used untill this full run of the screening platform and its validation it seems that while the fold difference between the N-repressed and induced luminesecence values was statistically significant (when analysed via an ANOVA), for the purposes of screening chemical libraries the difference was insuffcient. The reason the ANOVA test validated conditions that failed to satisfy the *Z* factor statistical parameter was due to differences in the nature of the testing methods. Both statisitcal tests take account for range and variation of data within treatment groups and then the difference between the groups being compared, however the *Z* factor statistical parameter is more stringent when classifying these statistical differences – a single outlier in a treatment group can have a strong negative effect on the *Z* factor regardless of the number of replicates involved (Zhang et al., 1999) . This is a desired trait in a statistical test when validating a screening platform as a chemical treatment is usually only applied once in a single setup and any background interference could mask the appropriate identification of an interaction. For this purpose the *Z* factor statistical

parameter has been used here to confirm that the N-regime conditions are sufficient in order to adequately screen chemical libaries for antagonists of N-repression.

This validation provides statistical assurance that whilst screening the chemical libraries for antagonists of N repression any positive influence on the luminescence output from N repressed samples is a result of the chemical treatment being applied and not influenced by the N treatment regime. The *Z* factor statistical parameter, as used in this research has been shown by other studies to be a suitable tool to assess a chemical genetics screen (Zhang et al., 1999; Serrano et al., 2015). The inclusion of Arg was deemed acceptable as *pNRT2.1::LUC* in the presence of low and high N conditions was both stimulated and repressed in the presence of Arg, respectively. As Arg results in the enhanced stimulation of the luminescence signal, yet does not interfere with its Gln repression it provides an opportunity to investigate chemical anatagonists of *pNRT2.1::LUC* in a statistically viable manner, as its inclusion allowed statistical validation by the *Z* factor statistical parameter. In addition, no exogenous Arg was present in the luciferase assay as the seedlings were placed in a fresh solution containing reaction buffer. This and a lack evidence from an extended literature search ruled out the possibility that Arg might be having a direct effect on the luciferase enzyme in the luciferase reaction to produce the enhanced luminescence signal.

4.2.7 Summary

In this chapter the individual steps of the protocol for the chemical genetics screening platform were successfully optimised to provide the most suitable conditions to perform the chemical screen. For the luciferase assay it was concluded that the combination of 1 mM luciferin and 4 mM ATP over a 24 h period provided the maximum luciferase output, with additional increases in reagent concentrations providing not further statistical improvement. Additionally, despite evidence provided in the literature on the beneficial properties of BSA in the luciferase assay it was concluded that its inclusion provided no statistical benefit for the luminescence output, and so was consequently ommited. Using the optimised luciferase assay, nitrate induction of *pNRT2.1::LUC* luminescence was optimised. These experiments concluded that

under the experimental conditions of this study 0.3 mM KNO_3 over a 24 h period resulted in the maximum induction of luminescence.

Initial experimentation on N-repression conditions concluded that 2 mM Gln applied simultaneously with 0.3 mM KNO_3 resulted in the lowest luminescence output. When the repressive effect of Gln (0.3 mM KNO_3 + 2 mM Gln) was compared to that of 19 other amino acids it was shown that overall Gln is as effective at repressing luminescence as the majority of other amino acids (Ala, Asn, Asp, Cys, Glu, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp and Val). During this investigation, the application of Arg (at 4 mM) had a strong stimulatory affect on luminescence when in the presence of nitrate only.

The final experiments in this chapter used the Z factor statistical parameter to statistically validate the screening platform. Using the optimised N conditions ascertained from previous experiments (Figs. 4.4 – 4.7) it was concluded that the differences between the N-repressed and induced values, along with the variation within each treatment group were unsutiable to satisfy the Z factor statistical parameter. The test was sucessfully validated once a combination of 0.3 mM KNO_3 + 4 mM Arg was used to induce *pNRT2.1::LUC* luminescence and an increased Gln concentration (10 mM) (in the presence of 0.3 mM KNO_3 + 4 mM Arg) was used for N-repression of *pNRT2.1::LUC* luminescence. With the protocol optimised and the screening platform validated the next step was to screen the chemical libraries in search of chemical anatagonists of *NRT2.1* N-repression.

Chapter 5. Screening the chemical libraries for antagonists of N-repression

In Chapter 4, having optimised the luciferase assay and determined appropriate N treatments, it was established that a statistically viable screening platform had been developed. With the Z factor statistical parameter satisfied, screening for antagonists of N-repression could commence. In this chapter, the use of the screening platform to screen the chemical libraries for antagonists of N-repression is described.

The first chemical library selection was the 'Library of AcTive Compounds on Arabidopsis' (LATCA) compiled of compounds in the LOPAC, Spectrum and Chembridge libraries that displayed pharmacological interactions with Arabidopsis seedling development, compounds in the Maybridge library with known bioactivity in *S.cerevisiae* and an additional selection of herbicides, common inhibitors, plant hormones, research chemicals and bioactive compounds. The second chemical library selection was the LOPAC library containing pharmacologically active compounds, focused on bioactivity in mammals, including pharma-developed tools, inhibitors, receptor ligands, approved drugs with known bioactivity in most signalling pathways and a combination of all major drug target classes. The third chemical library selection was the Spectrum collection containing the MicroSource Natural Product and Discovery libraries and compounds from the US and International Drug collections; containing a wide variety of biologically active and structurally diverse compounds it is intended for use with target specific assays.

5.1 Results
5.1.1 Screening the 'Library of AcTive Compounds on Arabidopsis' (LATCA) for antagonists of N-repression

The screening platform layout was designed so that 80 individual chemicals could be screened per plate (assigned to columns 2-11 of a 96-well plate). Samples

that were chemically treated were also N-repressed with 10 mM Gln, in the presence of 4 mM Arg and 0.3 mM KNO_3. The remaining samples at the ends of the plate (columns 1 and 12) were positive and negative controls for the experiment, induced (0.3 mM KNO_3 and 4 mM Arg) and uninduced (background N, 50 μM NH_4NO_3), respectively. Chemical compounds were tested in duplicate plates, screening for an interaction in both replicates, however if an interaction was present in one replicate only, the chemical would be recorded.

Screening of the LATCA chemical library yielded no chemicals that antagonised N-repression of luminescence, in either one or both of the replicates (data not shown). The presence of chemical compounds from the LATCA library did increase the variation within N-repressed samples, in comparison to observations where no chemicals were applied previously. This variation was relatively low and is not likely to have masked a chemical interaction.

5.1.2 Screening the 'Library of Pharmacologically Active Compounds' LOPAC for antagonists of N-repression

In the next phase of the screening programme, the 1280 pharmacologically active compounds from the LOPAC collection were tested using the same methodology as described above. In this case, one of the 1280 small molecules was found to be associated with an increased luminescence signal in both replicate plates (Fig. 4.1). In both replicates the signal was significantly greater than the other N-repressed samples on the same plate based on an ANOVA test ($p < 0.05$). The small molecule responsible for this effect was identified as camptothecin (CPT). CPT is a plant derived cytotoxic alkaloid that targets topoisomerase I (Vos et al., 2011, Shafiq et al., 2017).

A second chemical on the same plate, 2-chloroadenosine triphosphate tetrasodium also gave a significantly increased luminescence signal above the other N-repressed samples in one replicate (Fig 4.1A), but this effect was not reproduced in the other replicate (Fig 4.1B).

5.1.3 Screening the 'Spectrum collection' chemical library for antagonists of N-repression

Screening the Spectrum collection yielded two chemicals that antagonised N-repression of luminescence, however for both chemicals an effect was seen in only one of the duplicate plates. Aristolochic acid was identified in one of the replicates to have a significant interaction, antagonising N-repression (Fig. 4.2A). In the second replicate however, luminescence was not significantly different from the other N-repressed samples (Fig. 4.2B)

Aminocyclopropanecarboxylic acid (ACC) was the second chemical identified in the Spectrum collection. It had a significant interaction, antagonising N-repression in only one replicate (Figure 4.3A), However, again no significant effect was seen in the replicate plate (Fig. 4.3B).
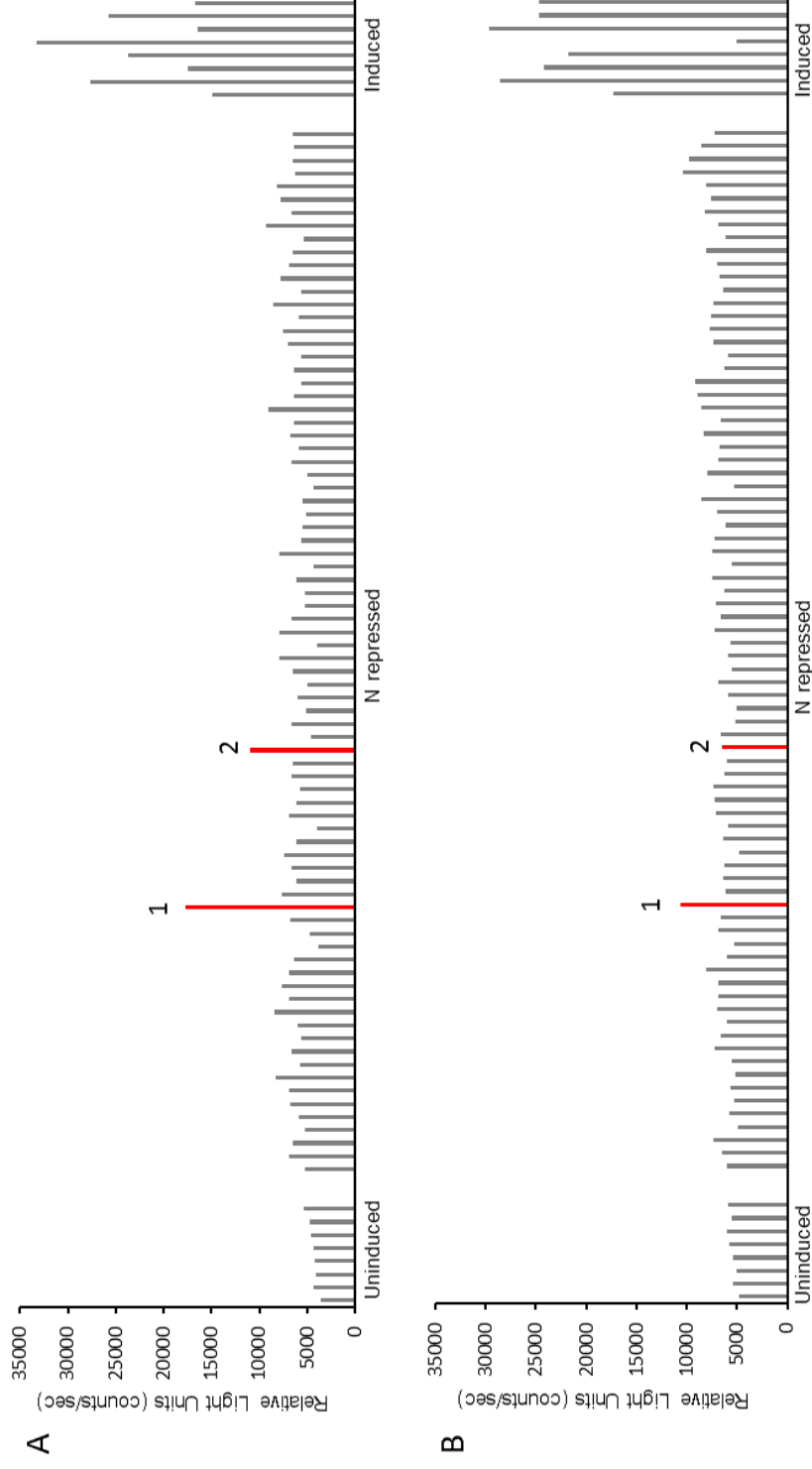
Figure 5.1 Screening for chemicals of interest in the LOPAC library, library plate number 5. Fourteen day old *pNRT2.1::LUC* seedlings were grown using the luciferase screening platform setup (Chapter 2.6.1), treated on 12 days with an initial concentration of 25 µM of each chemical in the well from the LOPAC chemical library and on 13 days treated with N applications: Uninduced (background N, 50 µM NH$_4$NO$_3$); N repressed (0.3 mM KNO$_3$ + 4 mM Arg, 10 mM Gln); induced (0.3 mM KNO$_3$ + 4 mM Arg). Samples that were treated with chemical library compounds were N repressed. Each data point is an individual sample (5-6 seedlings), with 8 samples uninduced, 80 samples N repressed and chemical treated and 8 samples induced, in two replicate plates (A & B). Bars highlighted in red show antagonist effect of chemical: 1) camptothecin; 2) 2-chloroadenosine triphosphate tetrasodium. Luciferase assay contained 4 mM ATP and 1 mM LUC and was performed in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6)
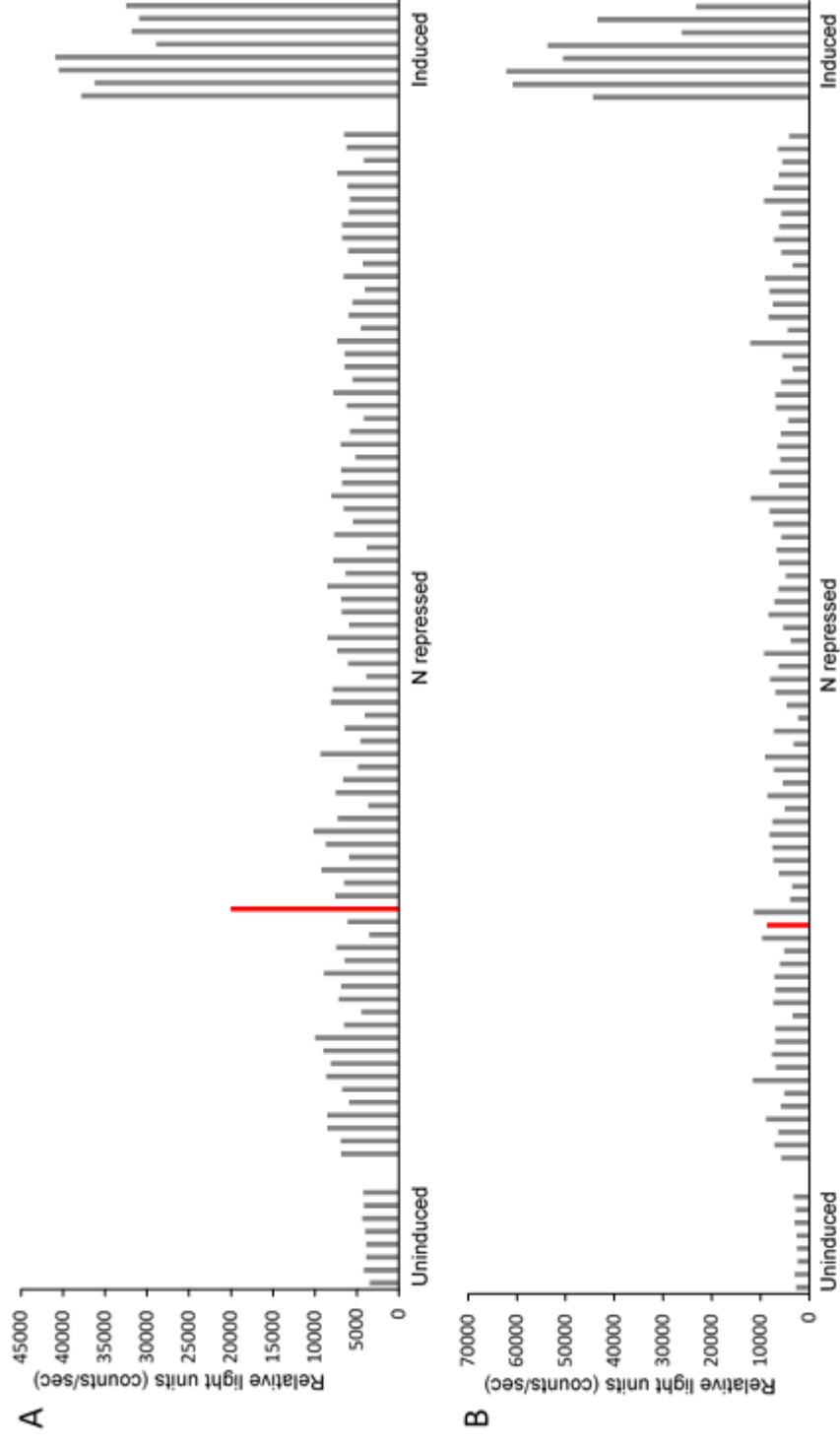
Figure 5.2 Identification of chemicals of interest in the Spectrum collection, library plate number 21. Experimental conditions for screening the Spectrum Collection were the same as for the LOPAC library as described in Fig. 5.1. The bar highlighted in red show antagonist effect of aristolochic acid (ACC).
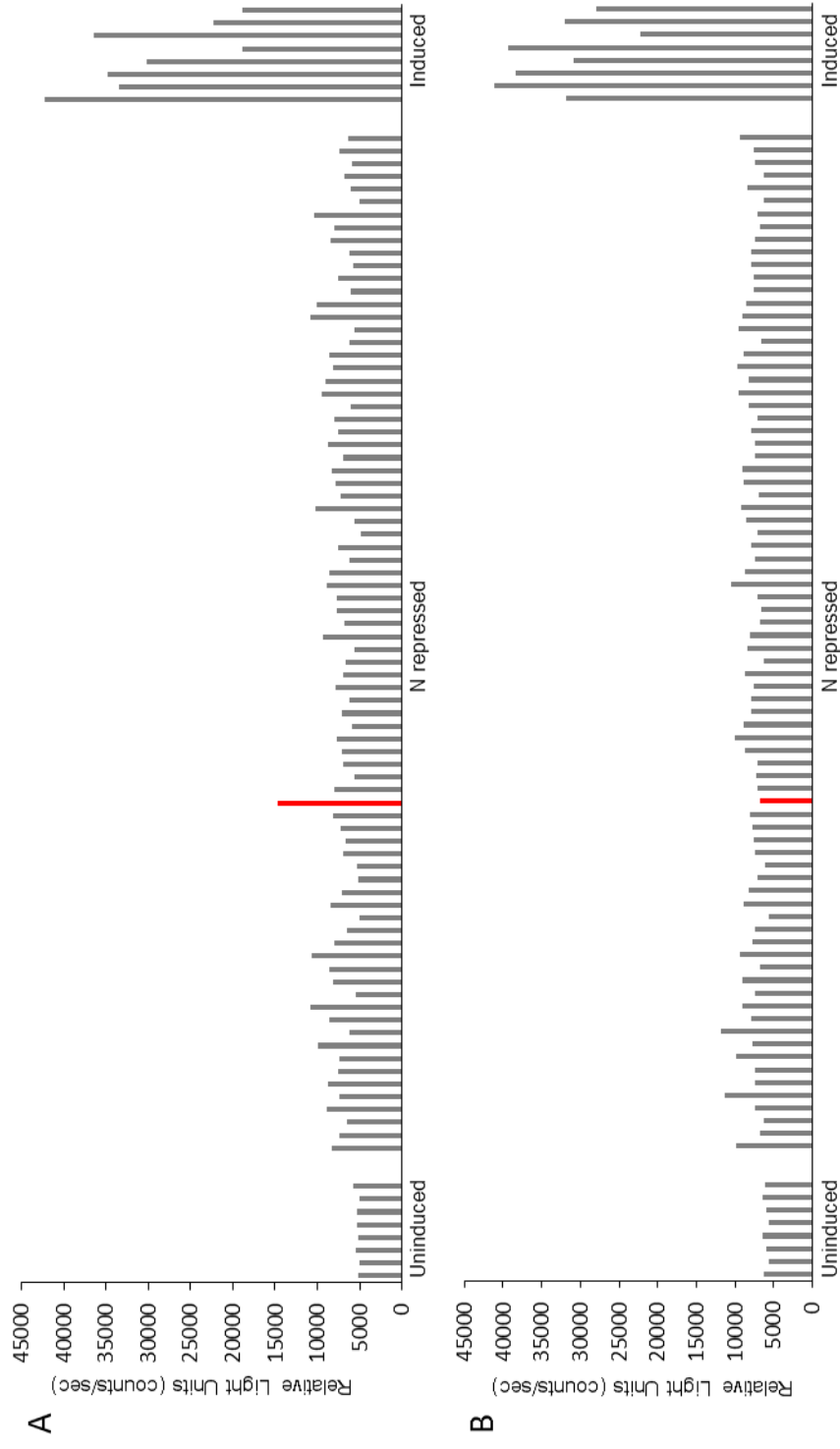
Figure 5.3. Identification of chemicals of interest in the Spectrum collection, library plate number 22. Experimental conditions for screening the Spectrum Collection were the same as for the LOPAC library as described in Fig. 5.1. The bar highlighted in red show antagonist effect of aminocyclopropanecarboxylic acid.

5.2 Discussion

As reported in the literature review previous research is yet to uncover many of the regulatory mechanisms behind N-repression of *NRT2.1*. So far studies in the field of genetics have employed traditional approaches to identify potential regulatory factors of *NRT2.1* N-repression. This chapter presents the first recorded use of a chemical genetics approach to dissect the N signalling pathway in plants. Screening three well known chemical libraries (the LATCA, the LOPAC and the Spectrum collection) containing molecules with known bioactivity in plants it is reported that one chemical (Camptothecin) acts as an antagonist of *NRT2.1* repression. This result was identified in both replicates and presents a novel discovery for what is known about N signalling and N-repression of *NRT2.1*. In addition, two other chemicals (2- chloroadenosine triphosphate tetrasodium and ACC) were reported as potential antagonists of N-repression, as the antagonist effect was only observed in one of the replicate plates for both chemicals. In this section these results will be discussed in greater detail in relation to the current understanding of N signalling in plants.

5.2.1 Identification of camptothecin as an antagonist of N-repression

A total of 7420 molecules were screened in duplicate from three different libraries using the screening protocol developed in the previous two chapters of this thesis. Out of those 7420 molecules only one firm hit was identified based on a strong signal from both replicate plates. This indicates a hit rate of only 0.013%, although this is a slight underestimate of the hit rate because there is an overlap between the LATCA library and the other two. One strong hit was identified, its presence enhanced the luminescence signal in roots growing under N-repressed conditions in both replicate plates. This compound from the LOPAC library was identified as (S)-(+)-camptothecin (CPT), a plant derived cytotoxic alkaloid that targets topoisomerase I. Topoisomerase I is known for relieving torsional stress created during the replication and translation of DNA (Vos et al., 2011, Shafiq et al., 2017). During these processes the rotation of DNA before the replication fork creates a positive supercoil, where the DNA is wound tightly and a negative supercoil before it, as the DNA is loosely wound together. It is the job of

topoisomerase enzymes in eukaryotic organisms to cut and re-ligate DNA in this supercoiled regions, to relieve the torsional stress created. The extent to which supercoiling takes place influences the organism's ability to regulate gene expression in those region for cells affected. Topoisomerase I is an essential enzyme in genetic replication and is evolutionarily conserved between Arabidopsis, rice, yeast and some mammals (i.e. humans and mice) (Shafiq et al., 2017, Champoux, 2001).

CPT targets the topoisomerase I-DNA transient complex, stabilising this intermediate and so blocking DNA regulation. In human topoisomerase-I the sensitivity of the CPT interaction is influenced by Arg364, Asp533 and Asn722, amino acids that are also highly conserved in plants (Shafiq et al., 2017). Observations of mutant topoisomerase-I Arabidopsis lines report that specific developmental phenotypes occur when the *Top1α* paralog is disrupted (e.g. malfunction of the meristem). However, when both paralogs are mutated the combination is lethal (Liu et al., 2014). Topoisomerase I has been shown to play an essential role in DNA replication, recombination and repair and chromatin remodelling (Vos et al., 2011, Pikaard and Scheid, 2014, Liu et al., 2014, Durand-Dubief et al., 2010). The involvement in chromatin remodelling provides an exciting development of our understanding of the role of topoisomerase I in the epigenetic processes that regulate gene expression.

5.2.2 Assessment of partially identified compounds that might antagonise N-repression

Partially identified compounds, where only one of the replicates showed an enhanced signal were also reviewed for potential interest. While it is highly unlikely a chemical interaction would only occur in one, not both replicate plates, it is possible that very occasionally there might be a complication that interferes with the interaction or signal detection. For this reason the identity and properties of the three molecules were examined in case some common features were found that would encourage further study.

The first partial identification was 2- chloroadenosine triphosphate tetrasodium, which is a purinergic G protein-coupled (P2Y) receptor agonist. Numerous P2Y receptors have been identified in mammalian tissues. However,

extensive searches have not identified any homologues in plant organisms (Roux et al., 2006, Ralevic and Burnstock, 1998). Additional research in the future would benefit from checking this result, in order to confirm whether an interaction is present or not. The second partial identification was aristolochic acid, a phospholipase A_2 inhibitor in neutrophils and a carcinogen, specifically targeting kidney cells in animal models (Chen et al., 2006, Gokmen et al., 2013). The third partial identification was ACC, which is an intermediate in the biosynthesis of the plant hormone ethylene (Van de Poel and Van Der Straeten, 2014). Given that there is a relationship between ethylene and N signalling in plants (Van de Poel and Van Der Straeten, 2014) this molecule could be of interest. Zheng et al. (2013) in Arabidopsis have shown that the up-regulation of *NRT2.1* in response to low N conditions stimulated ethylene biosynthesis. This stimulation of ethylene production in turn resulted in the repression of *NRT2.1*, and consequently the transporter function of NRT2.1. This implicates the ethylene signalling pathway in the tightly regulated *NRT2.1* response to high N conditions (Zheng et al., 2013). This interaction between the N and ethylene signalling pathways should be confirmed by future studies, under the same conditions used in this research to confirm this partial identification. However, in this research project, as the interaction was only identified in one of the replicate plates it was not prioritised for investigation.

5.2.3 Summary

Chapter 5 reports the first documented use of a chemical genetics approach to dissect the nitrogen signalling pathway in plants. Using the developed FrameStrip screening platform and 'ice capture' method in conjunction with the *pNRT2.1::LUC* Arabidopsis reporter line three chemical libraries (the LATCA, LOPAC, Spectrum collection) were screened for chemical antagonists of *NRT2.1* N-repression. Camptothecin, a molecule from the LOPAC library was identified in both screening platform replicates as a chemical antagonist of *NRT2.1* N-repression. The known target of camptothecin is Topoisomerase I, which has been shown to play an essential role in DNA replication, recombination and repair and chromatin remodelling (Vos et al., 2011, Pikaard and Scheid, 2014, Liu et al., 2014, Durand-Dubief et al., 2010). The involvement of topoisomerase I in chromatin remodelling provides an exciting development of our understanding of

the role of topoisomerase I in the epigenetic processes that regulate gene expression, in particular the regulation of *NRT2.1* in relation to the N status of the plant.

There were two other molecules which were partially identified as chemical antagonists of *NRT2.1* N-repression in the chemical screen. The first was 2-chloroadenosine triphosphate tetrasodium, a P2Y receptor agonist from the Spectrum collection which was partially identified as an antagonist of N-repression. However, as an effect was only seen in one of the screening replicates and there are no known homologues targets in plants no further investigation was undertaken for this molecule. The second partially identified chemical antagonist was ACC, a precursor to the plant signalling hormone ethylene. This molecule is of relevance to N signalling, as has been shown by studies such as Zheng et al. (2013) who demonstrated that the production of ethylene results in the repression of *NRT2.1*. Given its partial identification in this screen, ACC was not prioritised for investigation, however given additional time for further research it would be worth while running this chemical screen again under these experimental conditions to the interaction.

Chapter 6. Investigation of camptothecin (CPT) as an antagonist of N-repression

In Chapter 5, the LATCA, LOPAC and Spectrum Collection chemical libraries were screened for antagonists of N-repression, with a strong interaction being identified for camptothecin (CPT) in the primary screen. CPT is known to target topoisomerase I in both plants and mammals, but there were no previous reports of topoisomerase I being involved in N signalling in plants.

The first objective in this chapter was to confirm CPT's activity as an antagonist of N-repression using the *pNRT2.1::LUC* reporter line in a dose-response experiment. The second objective was to use real-time PCR to study the expression of the endogenous *NRT2.1* gene, and other N-regulated genes, as a means to rule out the possibility that the effect was specific to the *pNRT2.1::LUC* construct. A further objective was comparing the sensitivity of primary root growth to CPT in wild-type and three topoisomerase I mutant lines.

6.1 Results

6.1.1 Confirmation of CPT's ability to relieve N-repression of *pNRT2.1::LUC* expression

The effect of a range of CPT concentrations on *pNRT2.1::LUC* expression in the N-repression assay is shown in Fig. 6.1. As identified in the primary screen, CPT is able to antagonise N-repression, significantly increasing luciferase activity above the N-repressed samples without CPT. The antagonistic effect was not significantly different over the entire range of concentrations tested (from 25 nM to 50 µM), showing that CPT, even at very low concentrations has a significant antagonistic effect on N-repression. However, even at the highest CPT concentrations, expression of the *pNRT2.1::LUC* construct was not as high as in the nitrate-induced controls.
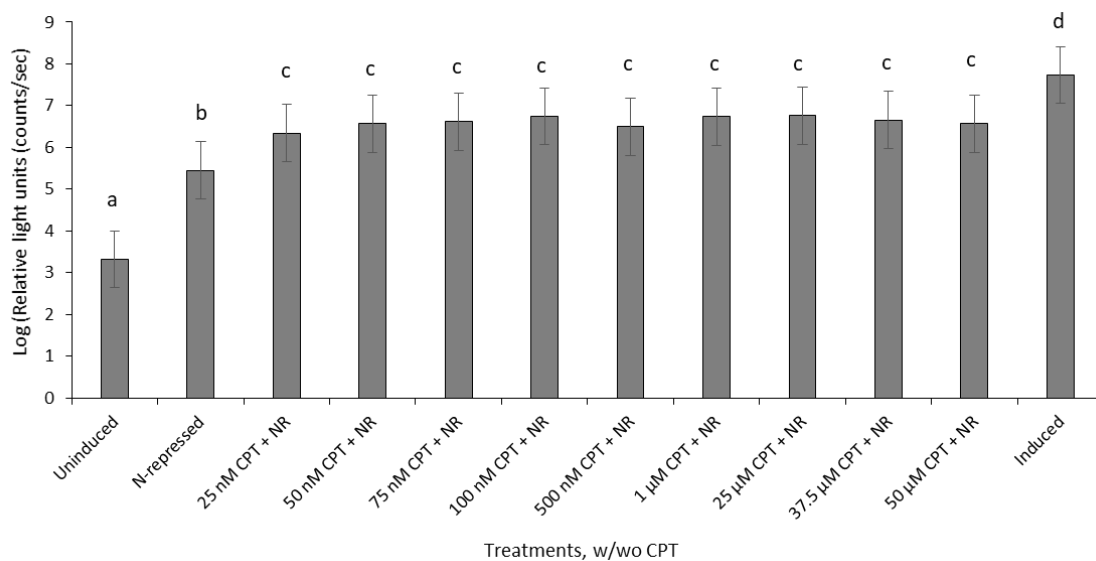
Figure 6.1 The effect of a range of concentrations of CPT on *pNRT2.1::LUC* expression under conditions of N repression Thirteen day-old *pNRT2.1::*LUC seedlings, grown in the luciferase screening platform (Chapter 4) were subjected to different N treatments: uninduced (background N, 100 μM NH_4NO_3); N-repressed (NR) (0.3 mM KNO_3 + 4 mM Arg, 10 mM Gln); induced (0.3 mM KNO_3 + 4 mM Arg). In the case of the 'N-repressed' treatments, a range of concentrations of CPT (0 to 50 μM) were applied 24 h before the N treatments were initiated. Luciferase assay performed in SpectraMaxi3 luminometer (Section 2.7.7). Values are log transformed means ± LSD (n = 32, 5-6 seedlings per tube) and different letters denote statistically significant differences by ANOVA (P<0.05, 340 degrees of freedom, LSD (5%) = 0.68).

6.1.2 Investigating the endogenous expression of *NRT2.1* and other N-related genes under different N-conditions and with CPT.

NRT2.1, *NRT2.2*, *NIA1*, *NIA2*, *NRT1.1*, *GS1-1* and *GS1-2* genes were selected for real-time PCR experiments as they are major components in the N uptake, assimilation and signalling pathways in Arabidopsis. Additionally, *pNRT2.1::LUC* was included so as to correlate the findings with the luciferase assays. Normalised relative quantification was calculated by normalising the targeted genes to two housekeeping candidates, actin and an uncharacterised protein (Kudo et al., 2016). These housekeeping genes have been shown to be more

stable under a broad range of conditions compared to other commonly used reference genes utilised in real-time PCR analyses (Kudo et al., 2016).

The results of the real-time PCR experiment are shown in Fig. 6.2. Nitrate strongly induced the expression of *pNRT2.1::LUC*, *NRT2.1*, *NRT2.2*, *NIA1*, *NRT1.1* and *GS1-2* genes, significantly increasing transcript levels. In particular, *pNRT2.1::LUC*, *NRT2.1*, *NRT2.2* and *NIA1* were most strongly stimulated. Conversely, *GS1-1* and *NIA2* showed no significant differences in transcript levels between the uninduced and nitrate induced conditions. Using Gln in the presence of nitrate to elicit N-repression showed a strong significant reduction in *pNRT2.1::LUC*, *NRT2.1*, *NRT2.2* and *NIA1* transcripts, compared to the solely nitrate induced. Conversely, the presence of Gln did not significantly alter *NRT1.1*, *NIA2*, *GS1-1* or *GS1-2* expression.

Analysis of gene expression in the presence of CPT confirmed that CPT had the effect of at least partially alleviating Gln-mediated N-repression in all four genes that were nitrate-induced and Gln-repressed (*NRT2.1*, *pNRT2.1::LUC*, *NRT2.2* and *NIA1*). Surprisingly, CPT also stimulated the expression of *NIA2* in the presence of nitrate and Gln, even though *NIA2* was neither nitrate-induced nor Gln-repressed in this experiment. *NRT1.1* was repressed only when both Arg and Gln were present and CPT also alleviated this repression. *GS1-1* was not repressed by any of the amino acid treatments and CPT had no significant effect on expression. However, in the presence of nitrate, Gln and Gln with Arg resulted in the stimulated expression of *GS1-1* which was only significantly different compared to the uninduced. *GS1-2* was repressed by all treatments, including the nitrate-induction and amino acid treatments with CPT.

It was unexpected that the addition of Arg to nitrate induction resulted in the repression of *pNRT2.1::LUC* as it strongly stimulated the luciferase signal in the luciferase screening platform. *NRT2.2* was also shown to be strongly repressed by the presence of Arg in nitrate induction conditions. Analysis of *NRT2.1* showed no significant difference in transcript levels between induction with and without Arg, yet the $Log_2(1/NRQ)$ value was slightly lower than nitrate alone, however this reduction was not to the extent of *pNRT2.1::LUC* or *NRT2.2*. No significant changes were seen for *NIA1*, *GS1-1* or *GS1-2*, but a strong stimulatory effect was observed for the *NRT1.1* expression in nitrate conditions including Arg. In agreement with the observations of Chapter 4, the addition of

Arg to Gln-repressed samples did not alter N-repression of *pNRT2.1::LUC* and *NRT2.1* expression significantly. Similarly, this effect was the same for *NIA1, NIA2, GS1-1* and *GS1-2*. Interestingly, the addition of Arg significantly reduced *NRT1.1* repression, when Gln-repression alone failed to alter expression compared to the nitrate induced. *NRT2.2* displayed a significantly increased expression with the addition of Arg, this was unexpected as Arg did not enhance the *NRT2.2* transcript, rather it significantly repressed it when included in nitrate induction conditions. Interestingly, while both show that the addition of Arg did not significantly affect the antagonistic effect of CPT, the mean $Log_2(1/NRQ)$ values were lower in the presence of Arg, following the trend observed for nitrate induction including Arg.
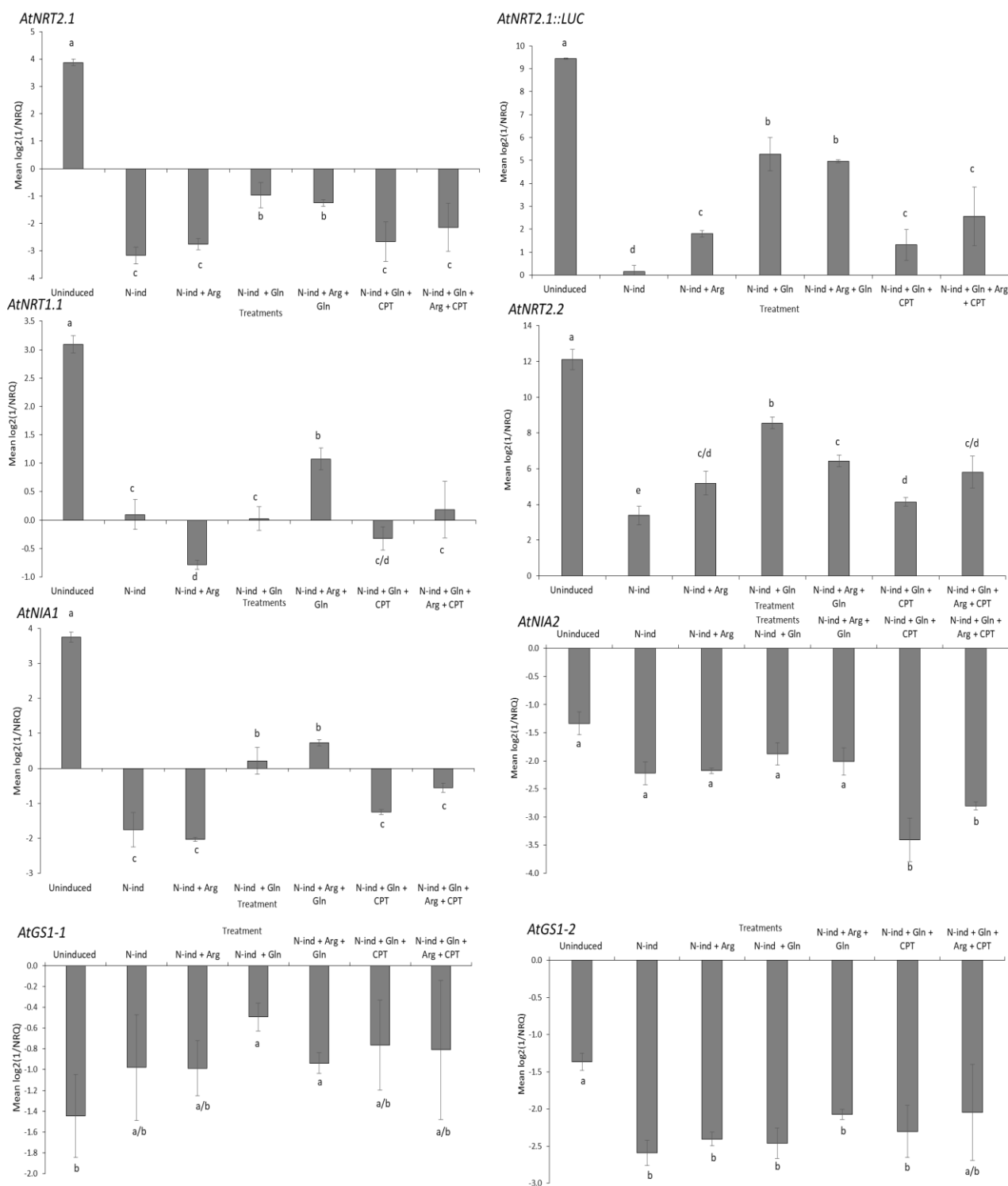
Figure 6.2. Real-time PCR analysis of expression of genes related to N signalling under various N treatments and in the presence of CPT. *pNRT2.1::LUC* seedlings were cultured using the FrameStrip deep-well method with a background concentration of 100 µM NH_4NO_3. Where used, CPT was applied at 100 µM to the wells, to 12 day old seedlings and N treatments were applied a day later. Treatments were: N-ind (induced, 0.3 mM KNO_3); N-ind + Arg (0.3 mM KNO_3 + 4 mM Arg); N-ind + Gln (0.3 mM KNO_3 + 10 mM Gln); N-ind + Arg + Gln (0.3 mM KNO_3 + 4 mM Arg + 10 mM Gln); N-ind + Gln + CPT (0.3 mM KNO_3 + 10 mM Gln + 100 µM CPT); N-ind + Gln + Arg + CPT (0.3 mM KNO_3 + 4 mM Arg + 10 mM Gln + 100 µM CPT). Mean Normalised Relative Quantification (Log$_2$ (1/NRQ)) values shown ±SE (n= 3 independent replicates, root material for each was from a separate deep-well plate) where negative fold changes represent upregulation and positive fold changes represent downregulation of the gene. Different letters denote statistically significant differences identified by ANOVA (p<0.05, 20 degrees of freedom, LSD (5%) = 1.15 (*NRT2.1*), 1.51 (*NRT2.1::LUC*), 0.55 (*NRT1.1*), 1.35 (*NRT2.2*), 0.75 (*NIA1*), 0.94 (*NIA2*), 1 (*GS1-1*), 0.72(*GS1-2*)) on Log$_2$(1/NRQ) log transformed data.

6.1.3 Using root growth of topoisomerase I Arabidopsis mutants as a proxy for CPT inhibition sensitivity

CPT is known to act as an inhibitor of seedling growth, with root growth being particularly sensitive (Tao and Buta, 1986). If this effect is due to CPT's targetting of topoisomerase I, then it would be expected that topoisomerase I mutants would show reduced sensitivity to the inhibitor. To determine the effect of CPT on primary root growth, wild-type and three topoisomerase I mutant Arabidopsis lines were grown on vertical agar plates containing a range of CPT concentrations (Fig. 6.3). The *top1α-1*, *top1α-2* and *mgo1-7* lines contained mutations in genes encoding topoisomerase type I, enzyme subtype A.

Primary root growth in the wild-type was sensitive to CPT application at 25 μM and 50 μM significantly inhibiting growth by 25% and 50% respectively. At 75 μM and above CPT reached a maximum effect, with no significant increase in root growth inhibition over 68%. The *top1α-1* and *top1α-2* mutant lines were less sensitive to CPT, with lower percentage root growth inhibition for each concentration, compared to the wild-type. Both mutants up to 75 μM CPT displayed approximately half the percentage of primary root growth inhibition of the wild-type. Even at 100 μM CPT, *top1α-1* and *top1α-2* displayed 49% and 63% root growth inhibition, respectively, compared to the 73% of the wild-type. Unexpectedly, at the highest concentration tested (100 μM) the *mgo1-7* mutant was almost as sensitive to CPT as the wild-type.
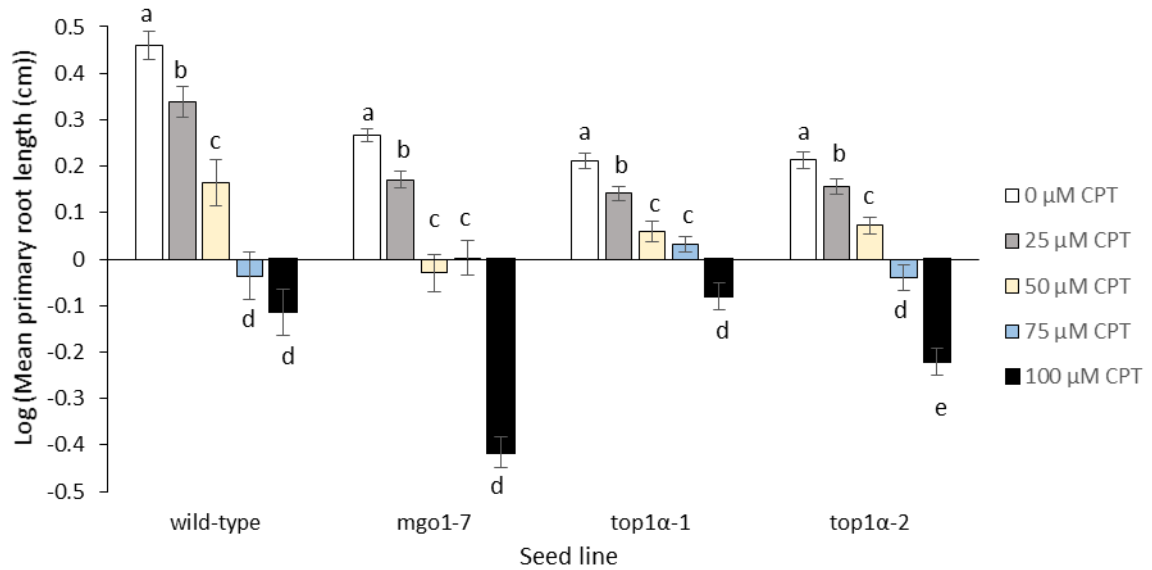
Figure 6.3 Using root growth of topoisomerase I Arabidopsis mutants as a proxy for CPT inhibition sensitivity Seedlings of wild-type and three topoisomerase I mutants (*mgo1-7, top1α-1, top1α-2*) were grown for 5 days on vertical agar plates (Section 2.5). Eight seedlings were selected for uniformity of primary root growth and transferred to new vertical agar plates containing 1 mM KNO_3 and CPT at the specified concentrations. The increase in primary root length over the following 7 days was measured. Values are log transformed means of primary root length (cm) ±SE (n = 8 individual seedlings). Different letters denote statistically significant differences identified within each seed line by ANOVA (P<0.05, 157 degrees of freedom, LSD (5%) = 0.08 (wild-type), 0.05 (*mgo1-7*), 0.05 (*top1α-1*), 0.06 (*top1α-2*).

6.2 Discussion

The identification of CPT as a chemical antagonist of *NRT2.1* N-repression in the chemical genetics screening platform was further investigated in this chapter. The first step was to confirm the effect of CPT on N-repressed *NRT2.1*-dependent luminescence in the *pNRT2.1::LUC* Arabidopsis reporter line. This was performed through a dose-response experiment to further validate the initial findings from the screen and to give an indication of the CPT dose effect. The next step was to confirm the interaction of CPT at the gene level, demonstrating that the results obtained from using the *pNRT2.1::LUC* reporter line and luciferase assay were representative of the endogenous expression of *NRT2.1*. The final step was to investigate the detrimental effects of CPT on Arabidopsis root growth, as CPT is known to be poisonous to plants, inducing apoptosis (Vos et al., 2011, Shafiq et al., 2017). In this section these results will be discussed in greater detail in relation to the current understanding of N signalling in plants.

6.2.1 CPT is confirmed to antagonise N-repression in the *pNRT2.1::LUC* line

CPT was shown in Fig. 6.1 to antagonise N-repression in the luciferase assay. This confirms the interaction observed in the primary screen, with CPT antagonising N-repression of luminescence significantly above the other N-repressed samples in both plates. Despite a range of concentrations being investigated, there was no significant difference in the antagonistic response, suggesting that even at the lower CPT concentrations investigated the effect on *NRT2.1* N-repression was at its greatest. Remarkably the antagonistic effect of CPT was seen even at very low concentrations. Extrapolating the data from the primary root growth response to CPT experiment in Fig. 6.1 suggests that concentrations as low as 25 - 75 nM would be below the threshold at which plant growth is inhibited. This powerful growth inhibition effect even at low concentrations has been seen in mammalian cells, where nM concentrations of CPT are cytotoxic for various tumour cell lines (Luzzio et al., 1995).

CPT is known as an inhibitor of topoisomerase I, specifically the B subtype which intercalates between the DNA cleaved ends in the enzyme active site during DNA replication. This results in the stabilization of the DNA-cleavage

complex leading to the ubiquitinylation and SUMOylation of topoisomerase I (Staker et al., 2002, Vos et al., 2011). Topoisomerase IB is associated with relieving torsional stresses of positively and negatively supercoiled DNA, whereas topoisomerase IA is only associated with the latter. At present no small molecules have been identified to target topoisomerase I A (Vos et al., 2011). This suggests that topoisomerase IB is the component, or is related to a mechanism responsible for eliciting N-repression. In other Arabidopsis research, antagonising topoisomerase I with CPT in this fairly general manner has been shown to disrupt specific elements of plant development, such as meristem development and phylotaxis   (Liu et al., 2014, Takahashi et al., 2002). Fig. 6.1 suggests that CPT, even at low concentrations is effective at antagonising topoisomerase I, likely causing changes in gene regulation that led to the antagonism of N-repression.

6.2.2 Investigating the endogenous expression of *NRT2.1* and other N-related genes under different N-conditions and with CPT

Real-time PCR experiments (Fig. 6.2) confirmed previous reports that high external N concentrations (in the organic or inorganic form) lead to repression of both the *NRT2.1* gene and the *pNRT2.1::LUC* transgene (Nazoa et al., 2003, Girin et al., 2007, Girin et al., 2010). These experiments were used to confirm that CPT was able to antagonise N-repression of *NRT2.1::LUC* as well as the endogenous *NRT2.1* gene, leading to increased accumulation of the respective mRNAs . Furthermore, CPT treatment also has a wider effect on N-repression in roots by enhancing the expression of two other genes in the nitrate assimilatory pathway that are regulated in a similar way to *NRT2.1, NRT2.2* and *NIA1*. Significantly, CPT had no positive effect on two genes whose expression is not N-repressed, *GS1-1* and *GS1-2*. However, surprisingly, CPT did stimulate the expression of *NIA2*, even though *NIA2* was not significantly repressed under high N conditions, suggesting that its expression is under negative control by a separate CPT-sensitive regulatory pathway.

Since CPT is best known for its ability to disrupt the activity of topoisomerase I (Vos et al., 2011, Staker et al., 2002, Liu et al., 2014), these

results suggest a role for topoisomerase I in the process of N-repression of genes of the N assimilatory pathway. Topoisomerase I is a key enzyme in DNA replication and transcription (hence the use of CPT as anti-tumour drug) and is responsible for relieving torsional stresses. These torsional stresses are produced by supercoiling of the DNA which occurs as the DNA is wound and unwound during replication and transcription (Staker et al., 2002). The topoisomerase I enzyme binds to the DNA double strand to form a topoisomerase I-DNA complex. Through transesterification it then ligates a single DNA strand, creating a break where one end is bound to the topoisomerase I-DNA complex and the other is free. The free end of the broken strand then rotates around the complimenting intact DNA strand, releasing the torsional stress on the DNA double helix. Once a single rotation of the broken strand has been made the topoisomerase I-DNA complex then relegates the two ends back together through transesterification. The topoisomerase I enzyme then dissociates from the DNA, ending the regulatory interaction (Fig. 6.4) (Staker et al., 2002; Liu et al., 2014).
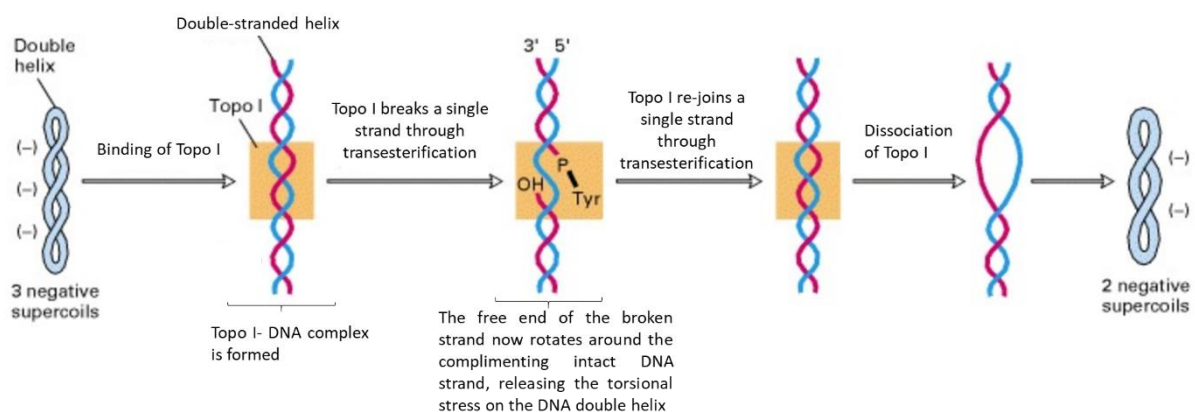
Figure 6.4 The regulatory role of topoisomerase I in relieving torsional stress on DNA Topoisomerase I (TopoI) is shown to bind to the DNA and relieve torsional stress from supercoiling via transesterification. Figure adapted from Lodish et al. (2000).

CPT is known to interact with topoisomerase I by binding to the topoisomerase I-DNA complex and preventing the relegation of the broken ends. This results in the topoisomerase I-DNA complex persisting on the stationary

broken strand of DNA. When DNA replication occurs, as the replication fork advances along the DNA molecule it collides with the topoisomerase I-DNA complex. This results in both strands of the DNA breaking apart and so the cell undergoes apoptosis (Staker et al., 2002; Liu et al., 2014).

As topoisomerase I is responsible for relieving torsional stresses, the findings from Fig 6.2 seem at first counter-intuitive. However, there are a number of recent studies indicating that topoisomerase I plays an important role in the epigenetic control of transcription in plants (Cao et al., 2015, Liu et al., 2014, Pikaard and Scheid, 2014, Sun et al., 2014). Nucleosomal spacing in gene regulatory regions has been shown to be regulated by *TOP1α*, with TOP1α/MGO1 and subunits of Polycomb Group Protein (PcG) interacting to deposit an epigenetic marker, H3K27me3 at target genes (Liu et al., 2014, Graf et al., 2010). Specifically, the Polycomb Repressor Complex 2 (PRC2) deposits the H3K27me3 histone marker which is identified by LIKE HETEROCHROMATIN PROTEIN1 (LHP1) in the Polycomb Repressor Complex 1 (PCR1) to establish repressive chromatin (Turck et al., 2007, Exner et al., 2009) occluding binding factors from the genes (Liu et al., 2014). Mutations in the *hni9* line were shown to be allelic to *INTERACT WITH SPT6* (*AtIWS1*), which encodes a protein in the RNA polymerase II complex. *HNI9/AtIWS1* was found to regulate several hundred N-responsive genes in Arabidopsis, including *NRT2.1* where repression of this gene by high N supply is associated with the accumulation of the H3K27me3 marker histone at the gene (Widiez et al., 2011). These studies and the present findings show that CPT is involved in regulating gene expression in relation to N-repression of *NRT2.1*.

6.2.3 Using root growth of topoisomerase I Arabidopsis mutants as a proxy for CPT inhibition sensitivity

It was expected that CPT would have a detrimental effect on root growth in the wild-type given its classification as a poison, targeting topoisomerase I as seen in Fig. 6.3 and ultimately leading to cell death. Primary root growth in all the investigated topoisomerase I mutants displayed reduced sensitivity to CPT although the *mgo1-7* mutant appeared to be more sensitive. Takahashi et al. (2002) reported a similar response for the *top1-α* lines when grown continuously

on the same range of CPT concentrations. They observed that roots were more sensitive to CPT exposure than in Fig. 6.3, with root inhibition being indistinguishable from the wild-type at 75 μM CPT. A key difference in experimental approach could account for this change, as Takahashi et al. (2002) germinated seed in the presence of CPT, rather than exposing established seedlings to the poison. This differentiates the experiments significantly as one is a CPT germination sensitivity assay, while the experiment in Fig. 6.3 is solely a root growth sensitivity assay.

The effect of CPT in the wild type was expected to be stronger than in the topoisomerase I mutants as the latter were disrupted in the *Top1α* paralog. This paralog, encodes TOP1α which has been shown to regulate specific development functions in plants, in both the root and the shoot (Zhang et al., 2016). It is thought that as CPT could not target this key regulatory enzyme and turn it into a DNA damaging agent the negative effect of CPT on root growth in the mutants was less than the wild type. The lack of the TOP1α paralog is not lethal in the Arabidopsis and seedlings can still grow and develop, even if stunted as TOP1β and topoisomerase II are still present to help relieve torsional stresses on the DNA. Topoisomerase II functions much the same as topoisomerase I, however instead of ligating and religating a single strand of DNA it is able to perform the same interaction on both strands of the DNA (Staker et al., 2002; Liu et al., 2014). Future research from this experiment could investigate the differences in DNA damage between the wild-type and mutant seed lines in the presence of CPT. A comet electrophoresis assay could be used to investigate DNA damage by looking for DNA double strand breaks and whether there is a difference between the wild-type and mutant seedlings after treatment with CPT.

6.2.4 Summary
In this chapter CPT's activity as an antagonist of N-repression in the *pNRT2.1::LUC* reporter line was confirmed. The dose-response experiment reported that CPT, even at very low concentrations has a significant antagonistic effect on N-repression. To investigate this further the effect of CPT was investigated at the gene level via qPCR, along with the different N treatment regimes used in this research. This experiment confirmed previous reports that

high external N concentrations (in the organic or inorganic form) lead to repression of both the NRT2.1 gene and the *pNRT2.1::LUC* transgene (Nazoa et al., 2003, Girin et al., 2007, Girin et al., 2010). In addition, two other genes that were responsive to the N-repression were also antagonised by CPT, *NRT2.2* and *NIA2.* This experiment also showed that the presence of Arg resulted in the repression of all genes, expect for *NRT1.1* where it stimulated expression. Given the stimulation of luminescence in the luciferase assay in the presence of Arg treatments, the repression of *pNRT2.1::LUC* was unexpected. In a final experiment root growth of topoisomerase I Arabidopsis mutants was used as a proxy CPT for CPT inhibition sensitivity. This experiment reported that CPT was detrimental to root growth in the WT, as was expected from it being a poison, targeting topoisomerase I. The higher the concentration of CPT applied, the stronger the inhibitory effect on root growth. Detrimental observations were made for root growth in the topoisomerase I mutants (*mgo1-7, top1α-1, top1α-2*), however they displayed a reduced sensitivity compared to the WT (namely *mgo1-7*), as expected. The differences in sensitivity between the WT and the topoisomerase I mutant lines provides further support for CPT targeting topoisomerase I and demonstrates that higher concentrations produce a stronger inhibitory effect. This experiment suggests that even at low concentrations of CPT, such as those being used in the chemical screen, the inhibitory effect on root growth is minimal, whilst the other experiments in this chapter have shown that such concentrations have a strong statistical effect on *NRT2.1* gene expression.

Chapter 7. General discussion

This research has for the first time adopted a chemical genetics approach to dissect the N signalling pathway in plants. The preliminary experiments detailed in Chapter 3 identified a suitable Arabidopsis reporter line, *pNRT2.1::LUC*, around which the FrameStrip and 'ice capture' methods were developed to establish a functional screening platform for this research. In Chapter 4 the protocols associated with this screening platform were then optimised, in order for the most suitable conditions for the luciferase assay and the N-treatment conditions to be established. These conditions were statistically validated by an appropriate test, the *Z* factor statistical parameter, providing a validation step to ensure that all aspects of the platform were viable for its use in a chemical genetics screen.

In Chapter 5 three libraries (LATCA, LOPAC, Spectrum collection) were then screened for chemical antagonists of *NRT2.1* N-repression, yielding one chemical, CPT which acted as a chemical antagonist in both screening platform replicates. CPT is known to target topoisomerase I in plants, which has been shown to play an essential role in DNA replication, recombination and repair and chromatin remodelling (Vos et al., 2011, Pikaard and Scheid, 2014, Liu et al., 2014, Durand-Dubief et al., 2010). The experiments detailed in Chapter 6 confirmed the antagonist effect of CPT in *pNRT::LUC* via a dose-reponse experiment, showing a strong statisitcal effect even at low concentrations. This antagonist effect was then confirmed at the gene level, using qPCR to investigate endogenous gene expression. This experiment reported that CPT indeed was an antagonist of *NRT2.1* expression under N-repression conditions. In addition, this experiment confirmed that the results from the luciferase assay in relation to different N treatments was indicative of the endogenous gene expression for *NRT2.1*. By also investigating additional genes associated with the N assimiltion and signalling pathways in plants a further insight into the regulation of *NRT2.1* under N-repression was produced. In a final experiment the detrimental effects of CPT on root growth were investigated using topoisomerase I mutants. The application of CPT was shown to inhibit root growht in a dose-dependent manner,

however the topoisomerase I mutants displayed a decreased sensitivity compared to the WT.

Given the findings of this research and the current understanding of N signalling in plants, N-repression of *NRT2.1,* the main topics and outcomes from this research will now be discussed in the following sections.

7.1 Evaluating the luciferase screening platform

This research established a novel chemical screening platform that identified camptothecin as a chemical antagonist of N-repression of *NRT2.1* and related genes. The FrameStrip method adopted here was designed for Arabidopsis, as is the case for many other screening platforms (Armstrong et al., 2004, Dejonghe and Russinova, 2017, Jan et al., 2004, Forde et al., 2013). However, the method is compatible with other seed types, providing they adhere to the 'minimum space' ethics of a screening platform. Characteristics that help determine suitability include: small seed size, ideally < 2 mm; seed that develop a root system gradually; seed that have a high germination percentage; seed populations that display uniformity of growth. As an example, a new screening platform, based on the FrameStrip method has demonstrated this using *Eragrostis tef* (Burrell et al., 2017).

By using a gene-based luciferase reporter system it is possible to perform a simple assay to gain a real-time quantitative insight into the transient expression of *NRT2.1* (Millar et al., 1992, Baldwin, 1996). This maintains the high-throughput principles of the screening platform, whilst allowing for many diverse classifications of molecules to be tested (Raikhel and Pirrung, 2005). Additionally, it means that the input of time and resources is only invested into chemicals that are influencing a specified gene, instead of a broader phenotype, such as changes in root architecture that is governed by multiple regulatory pathways. A recent investigation into the use of reporter lines within chemical genetics studies shows that almost 38% of the 40 studies selected used a reporter line for these reasons (Serrano et al., 2015, Burrell et al., 2017).

The luciferase screening platform maintained the setup simplicity associated with the FrameStrip method. Once assembled the platform is also self-sufficient until treatment application, except on day 7 when FrameStrips are raised up and assay plate nutrient solutions are replenished. The 'ice capture'

method maintains this principle, requiring no additional materials or equipment to generate and harvest root mass. A comparison with other platforms provides further support for these claims. The 'ice cap' genotyping platform (Su et al., 2011) requires a multiple stage setup procedure incorporating specialised growth plates, a pump-driven water hydroponics system and custom built racks to generate harvestable root material in a 96-well format. Another setup, using a plant chip for Arabidopsis phenotyping (Jiang et al., 2014) requires a highly specialised PDMS bespoke growth unit, employing a microfluidic system driven by syringe pumps and a hydroponic trapping method to sow seed to phenotype root architecture. Comparatively, the luciferase screening platform is made up of everyday lab consumables, except for the FrameStrip supports which were made on-site, simply by cutting sheets of acrylic (Fig. 2.4).

The luciferase screening platform could be used to investigate any suitable plant species with a luciferase reporter gene expressed in the root. It could also be used to investigate other aspects of N signalling, such as systematic N regulation. By applying different N treatments to the shoot and the roots and isolating the growth tube media from the assay plate media (by raising the FrameStrips up further) long distance signalling interactions could be investigated. In principle, this is similar to traditional split root experiments, where treatments are isolated to observe systemic regulation, however this would look at shoot to root derived signals. Other uses of the platform could be to generate root mass for genotyping, as a simplified, cost effective alternative to the 'ice cap' system (Su et al., 2011) or to investigate longer term root growth and development phenotypes in a semi-hydroponics context, using the deep-well plate setup (Fig. 2.7).

7.2 The effect of Arg on NRT2.1 expression

Observations with the *pNRT2.1::LUC* reporter line showed that Arg, in the presence of nitrate induction conditions strongly stimulated the luminescence signal. This increase was 3-fold higher than when nitrate alone was applied. An initial hypothesis for Arg stimulating *pNRT2.1::LUC* luminescence in the presence of low nitrate was a possible repression of NR activity, facilitated by a proposed increase in NO production. Luminescence observations also showed that the

stimulatory effect of Arg only occurred in the presence of nitrate, Arg alone did not stimulate luminescence. Based on this it was hypothesised that Arg, in the presence of nitrate was having a transcriptional effect on *pNRT2.1::LUC*, as the addition of Gln was able to repress luminescence, similarly to Gln-repression in the absence of Arg. Analysis of endogenous gene expression revealed that both *pNRT2.1::LUC* and *NRT2.1* expression were reduced by Arg in the presence of low nitrate conditions, however *NIA1* and *NIA2* expression were unresponsive. A study in barley reported that the exogenous application of Arg downregulated *NRT2.1* in the presence of nitrate (Zhuo et al., 1999) and decreased nitrate uptake in soybean and wheat (Muller and Touraine, 1992, Rodgers and Barneix, 1993). These findings show that the application of Arg, like many amino acids, is associated with N-repression of *NRT2.1*. However, interpretation of amino acid data must be treated with caution, as different amino acids are taken up and assimilated at different rates in the plant (Zhuo et al., 1999). Internal amino acid concentration analysis was performed in soybean, reporting that the endogenous application of Arg to roots led to a strong increase in Arg concentrations in planta (Muller and Touraine, 1992). A possible hypothesis for Arg stimulating luminescence in the presence of nitrate could be that Arg somehow enhances translation of the *NRT2.1* and *pNRT2.1::LUC* mRNA in this reporter line. This is supported by evidence in Arabidopsis that shows glucose stimulates NRT2.1 protein levels, independently of an effect on the *NRT2.1* expression (de Jong et al., 2014). Metabolites from the oxidative pentose phosphate pathway, along with glucose are known to directly stimulate *NRT2.1* expression, mediated by HEXOKINASE-1. It was reported that glucose was able to stimulate NRT2.1 protein abundance and activity independently of HEXOKINASE-1 mediated *NRT2.1* expression (de Jong et al., 2014). This provides a possible explanation for the stimulated luciferase output in the presence of low nitrate conditions and Arg. If Arg was able to stimulate luciferase protein abundance independent of the *pNRT2.1::LUC* transcript levels this would explain the results reported in this thesis.

7.3 Camptothecin as an antagonist of N-repression

Camptothecin is a plant alkaloid that binds to the DNA topoisomerase I-DNA complex, forming a transient intermediate that inhibits its activity (Hertzberg et

al., 1989). As discussed, topoisomerase I is involved in correcting torsional stresses of negative supercoiling created by RNA polymerase (Fig.6.4). This function is involved in DNA replication, recombination, repair and has also been shown to be involved in chromatin remodelling (Kieber et al., 1992, Vos et al., 2011, Pikaard and Scheid, 2014). There are number of recent studies indicating that topoisomerase I plays an important role in the epigenetic control of transcription in plants (Cao et al., 2015). For example, evidence has been reported that the Arabidopsis *TOP1α/MGO1* gene acts as a regulator of genome-wide nucleosomal spacing in gene regulatory regions (Liu et al., 2014), leading to a change in chromatin structure that could make promoters more accessible to transcription factors. It has been reported that TOP1α function reduces nucleosome density in most, if not all Arabidopsis genes, allowing binding factors access to them (Zhang et al., 2007, Liu et al., 2014). This suggests that the function of *TOP1α* might have a much larger role in all aspects of signalling and development than have been revealed. Supporting genetic evidence has shown that there are interactions between *TOP1α/MGO1* and subunits of the Polycomb Group Protein (PcG) (Graf et al., 2010). Of the complexes that make up PcGs, the Polycomb Repressor Complex 2 (PRC2) is known to deposit a repressive mark histone (H3K27me3) at target genes. This is identified by the Polycomb Repressor Complex 1 (PRC1) which interacts at the site to establish repressive chromatin (Sun et al., 2009, Sun et al., 2014). PcG target genes have been shown to have essential roles in plant development and are thought to be targeted due to the lack of a 5' nucleosome-free region, as seen in yeast, drosophila and the human genome (Lee et al., 2007, Mavrich et al., 2008, Schones et al., 2008). Furthermore, TOP1α appears to be important in floral meristems for promoting PcG-mediated histone 3 lysine 27 trimethylation (H3K27m3) at the *WUSCHEL* (*WUS*) gene (Liu et al., 2014). Thus, a picture is emerging of the importance of topoisomerase in chromatin remodelling and the regulation of the deposition of epigenetic marks. Against this background, the identification of topoisomerase I as a component in N repression of gene expression no longer appears surprising. Indeed, there is a clear link between this finding and the previous identification of the *HNI9* gene as corresponding to the *Arabidopsis INTERACT WITH SPT6* (*AtIWS1*) gene, which encodes a component of the RNA polymerase II complex (Widiez et al., 2011). Importantly repression of the *NRT2.1* transcription by high

N supply was shown to be associated with an HNI9/AtIWS1-dependent increase in histone H3K27m3 methylation at the *NRT2.1* locus (Widiez et al., 2011).

To further this research, expression of *NRT2.1* and other related genes should be investigated, using Arabidopsis topoisomerase I mutants (*top1α-1*, *top1α-2*, *top1β-1*, *mgo1-7*), under the treatment conditions used in this project. It is hypothesised that topoisomerase I mutants will display a desensitised response to high N-supply, based on the findings in the *hni9-1* mutant (Widiez et al., 2011) and the *NRT2.1* response to CPT (Fig. 6.2). Considering all the results, the exclusion of Arg may be relevant as, except for the luciferase data it was not reported to have a stimulatory effect on gene expression (except for *NRT1.1* in the presence of nitrate), as shown in other studies (Girin et al., 2007, Zhuo et al., 1999). Additionally, activity at the H3K27m3 histone should be investigated at the *NRT2.1* locus in these experiments and results should be compared to the reported findings of the *hni9-1* mutant (Widiez et al., 2011) to further our mechanistic understanding of N-repression. It would also be interesting to observe the effects of CPT in the absence of N treatments. Here we have shown that CPT antagonises N-repression of Gln alone and in the presence of Arg, however the effect of CPT alone was not investigated. It is hypothesised that CPT application could up-regulate *NRT2.1* expression alone, in the absence of N treatments. This is supported by recent evidence showing that CPT in Arabidopsis and mouse de-repressed RNA-directed DNA methylation of loci under transcriptional silencing by H3K9 or DNA methylation (Dinh et al., 2014, Huang et al., 2012). The increase in expression of these endogenous genes suggests that *NRT2.1* could also be affected in the same way.

7.4 Conclusions

This research documents for the first time a chemical genetics approach to investigate the N signalling pathway in Arabidopsis. The development of the FrameStrip platform and the 'ice capture' method provides a novel tool for the current field of plant science to further investigate signalling and regulatory components of the N signalling pathway. In this research the *pNRT2.1::LUC* reporter line, in conjunction with the screening platform was used to screen three chemical libraries containing molecules compiled for bioactivity and potential targets in plants. From this, CPT was identified as a novel chemical antagonist of

NRT2.1 N-repression. This discovery was validated at the gene level and was shown to influence a number of genes in the N signalling pathway which were sensitive to the N status of the plant (i.e. *NRT2.1*, *NRT2.2* and *NIA2*). CPT is classified as a poison, which targets and binds to topoisomerase I to form DNA topoisomerase I-DNA complex, forming a transient intermediate that inhibits its activity (Hertzberg et al., 1989). The proposed inhibition of topoisomerase I by CPT, resulting in the antagonistic response of *NRT2.1* N-repression suggests that its role as an epigenetic regulatory factor of gene transcription within plants is essential to the N signalling pathway in plants. This discovery enhances what is currently known about N signalling in plants, providing a new regulatory component in a complex and poorly characterised pathway. Topoisomerase I can now be targeted in future research in order to better understand its complete role in N signalling and to aid the search for other regulatory components of the N signalling pathway that have eluded research thus far.

7.5 Future research

The field of plant science would benefit from using the achievements and discoveries made in this research project to drive future research. Concerning the screening platform developed in this project, this novel tool provides an effective and convenient method to employ a chemical genetics approach in the study of any signalling pathway in plants, provided a suitable plant reporter line can be acquired. In terms of further N signalling research, it could be used to identify other chemical antagonists of N-repression in a similar manner to this project, in an effort to uncover yet more regulatory components of the pathway. Alternatively, the platform could be modified to investigate specific aspects of the pathway. By raising the base of the FrameStrip growth tubes up above the solution of the assay plate's wells the root material in each section could be isolated for individual N treatments. This approach could be used to further investigate shoot to root long distance N signalling in plants, which could be further challenged with a chemical genetics approach to dissect the pathway further.

Future research could also investigate the strongly stimulated luminescence signal from the *pNRT2.1::LUC* reporter line when Arg, in the presence of nitrate was applied for *NRT2.1* induction. Given that the qPCR data

showed that the addition of Arg did not stimulate *NRT2.1* expression at the gene level an experiment using isolated samples of luciferin substrate and luciferase enzyme could be set up with and without Arg in an attempt to replicate the stimulatory effect. This would help to determine if Arg influenced the luciferase assay itself, if not it could provide further support for the theory that it was a post-translational effect in planta, as proposed in the general discussion. Additional future experimentation would benefit from looking at the endogenous gene expression of *NRT2.1* under N-repressed conditions, in the presence of CPT, but without the presence of Arg. In this research the effect of CPT was only observed at the gene level in the presence of nitrate. Given that the inclusion of Arg had no effect on the *NRT2.1* transcript under N-repressed conditions it is unlikely to have contributed to the stimulatory response observed when CPT was included. However, further experiments would allow this to be confirmed.

Concerning the discovery of CPT as an antagonist of *NRT2.1*, a key future experiment would be to investigate the activity of the NRT2.1 transporter in the presence of CPT. As known from the literature, the function of the NRT2.1 transporter is under the transcriptional control of the *NRT2.1* gene (Krapp et al., 1998, Muller and Touraine, 1992). Given the results from the endogenous gene expression in this research, it is hypothesised that under N-repressed conditions in the presence of CPT, the repression of the NRT2.1 transporter function would also be antagonised. This could be observed by performing a N media depletion experiment, however given that the NRT2.1 transporter is responsible for both the influx and efflux of nitrate from the root experiments using N^{13} would be more suited. By using N^{13} NRT2.1 nitrate influx into the roots could be easily visualised in a much more reliable manner. A final recommendation for future research would be to investigate the endogenous expression of genes that were both N responsive and antagonised by CPT in this research, but only in the presence of CPT. It is hypothesised that CPT, in the absence of N-repression would still be able to stimulate *NRT2.1* expression given our current understanding of the regulatory component it targets, topoisomerase I.

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Appendix I. Investigating the effect of the timing and concentration of Gln treatment on repression of nitrate-induced *pNRT2.1::LUC* expression (non-transformed data)
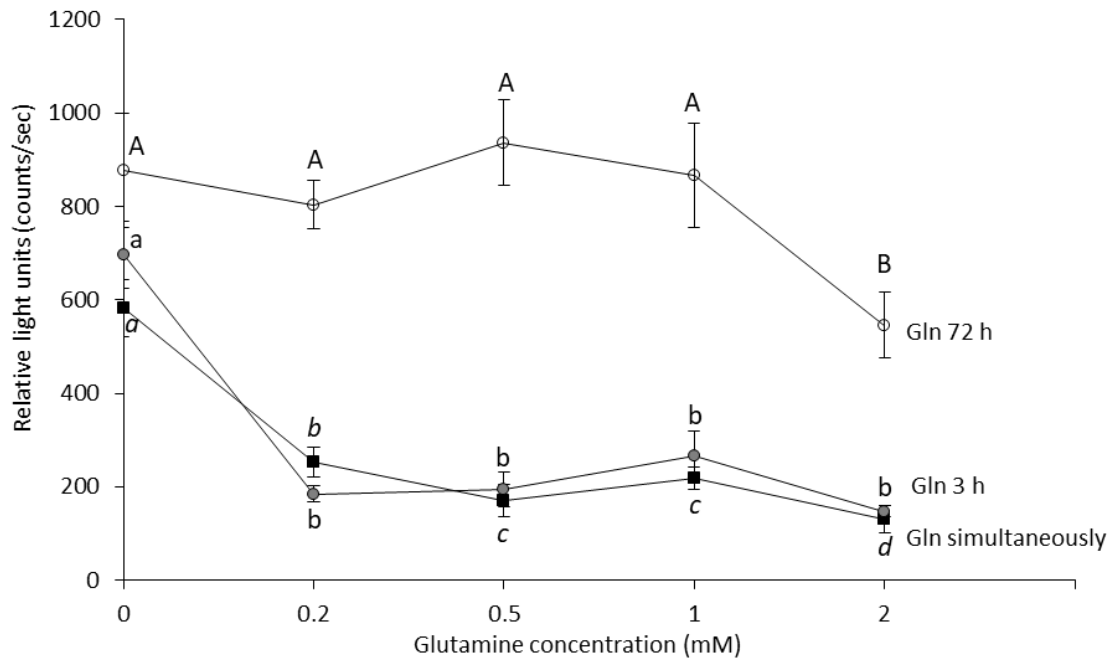

Figure 8.1 Investigating the effect of the timing and concentration of Gln treatment on repression of nitrate-induced *pNRT2.1::LUC* expression. Seedlings of the *pNRT2.1::LUC* line were cultured using the FrameStrip method in medium containing 50 μM NH_4NO_3 and nitrate induction was performed on 14 day old seedlings using 0.3 mM KNO_3. Gln treatments were applied at a range of concentrations either at the same time as the nitrate treatment (closed squares), 3 h in advance of the nitrate treatment (closed circles) or 72 h in advance of the nitrate treatment (open circles). Roots were harvested and frozen 24 h after the start of the nitrate treatments. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values means ± SE (n = 16) and different letters denote statistically significant differences (ANOVA, $p<0.05$, 145 degrees of freedom, LSD 5%) identified from log transformed values.

Appendix II. Comparison of the effectiveness of 19 different proteinogenic amino acids at repressing expression of nitrate-induced *pNRT2.1::LUC* (non-transformed data).
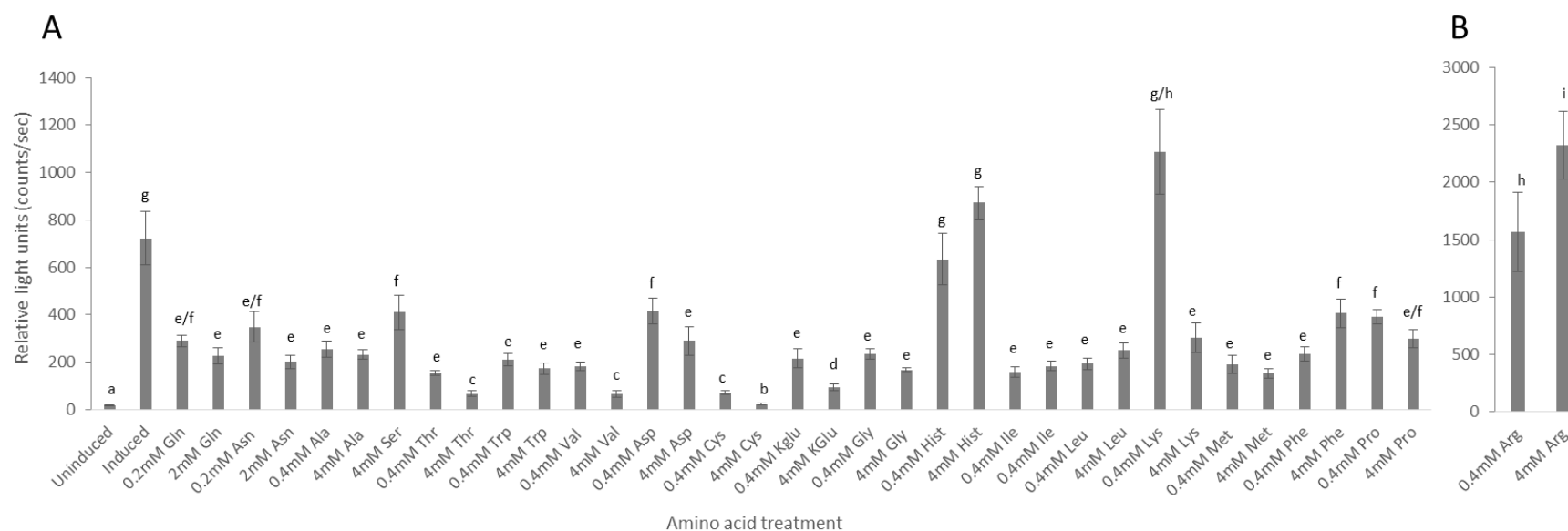

Figure 9.1 Comparison of the effectiveness of 19 different proteinogenic amino acids at repressing expression of nitrate-induced pNRT2.1::LUC. Seedlings of the pNRT2.1::LUC line were cultured using the FrameStrip method in medium containing 100 µM NH4NO3 and nitrate induction was performed on 13 day old seedlings using 0.3 mM KNO3. Individual amino acid treatments were applied simultaneously with the nitrate treatment for all amino acids (A) except Arg (B) and root material was frozen after 24 h. Luciferase activity was measured in root extracts using 4 mM ATP and 1mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values are means ± SE (n = 8) and different letters denote statistically significant differences (ANOVA, p<0.05, 281 degrees of freedom, LSD 5%) identified from log transformed data.

Appendix III. Investigating the stimulatory effect of Arg on *pNRT2.1::LUC* expression (non-transformed data).
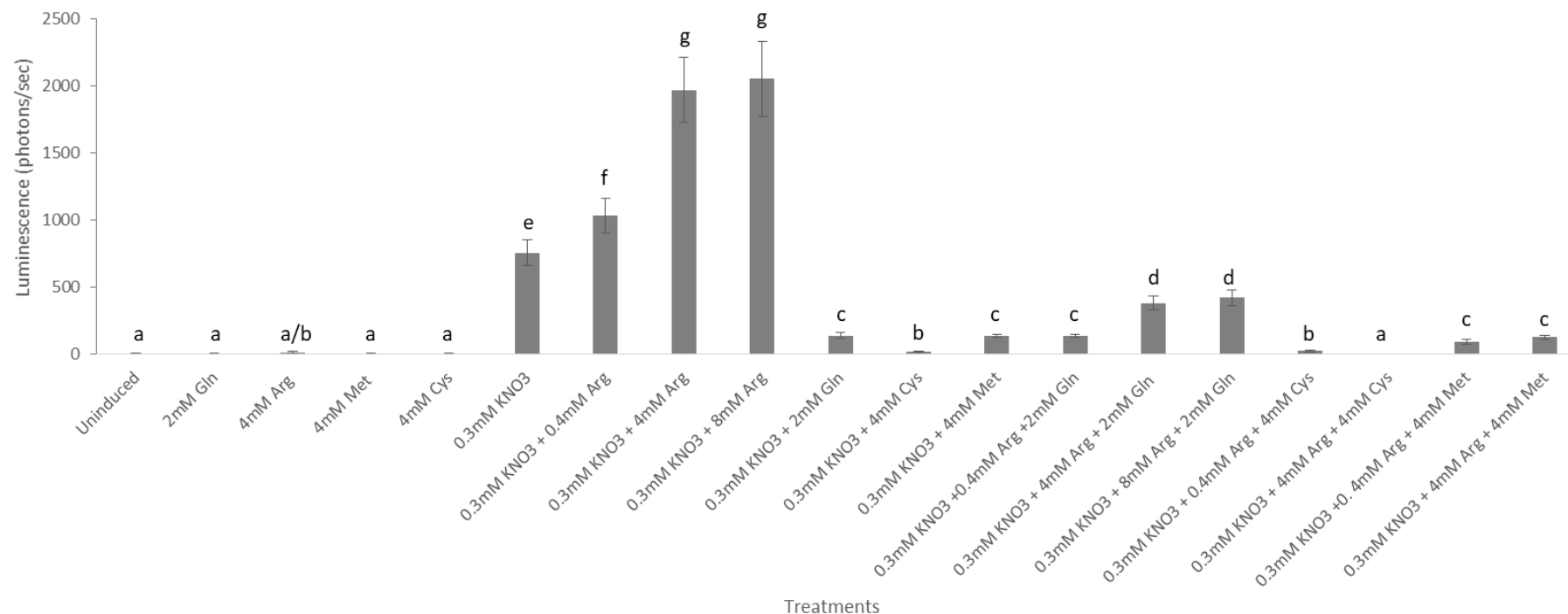

Figure 10.1 Investigating the stimulatory effect of Arg on *pNRT2.1::LUC* expression. Treatments were performed on 14 day old seedlings of the *pNRT2.1::LUC* line as for Fig. 4.8. Treatments with nitrate or amino acids alone, or combinations of nitrate and one or more amino acids as indicated in the Fig. 4.8, were applied simultaneously and roots frozen 24 h later. Luciferase activity was measured in root extracts using 4 mM ATP and 1 mM luciferin in a LUMIstar Omega, BMG dedicated plate reader (Section 2.7.6). Values are means ± SE (n = 8) and different letters denote statistically significant differences (ANOVA, p<0.05, 149 degrees of freedom, LSD 5%) identified from log transformed data.

Appendix IV. The effect of a range of concentrations of CPT on *pNRT2.1::LUC* expression under conditions of N repression (non-transformed data)
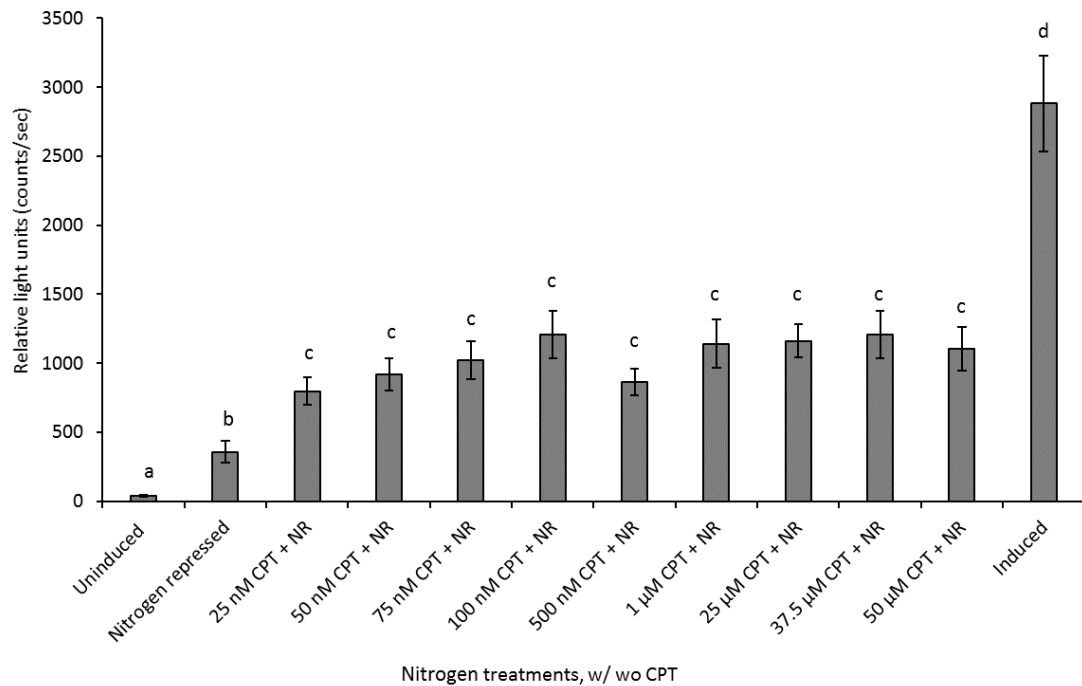

Figure 11.1 The effect of a range of concentrations of CPT on *pNRT2.1::LUC* expression under conditions of N repression Thirteen day-old *pNRT2.1::*LUC seedlings, grown in the luciferase screening platform (Chapter 4) were subjected to different N treatments: uninduced (background N, 100 µM NH_4NO_3); N-repressed (NR) (0.3 mM KNO_3 + 4 mM Arg, 10 mM Gln); induced (0.3 mM KNO_3 + 4 mM Arg). In the case of the 'N-repressed' treatments, a range of concentrations of CPT (0 to 50 µM) were applied 24 h before the N treatments were initiated. Luciferase assay performed in SpectraMaxi3 luminometer (Section 2.7.7). Values are ± SE (n = 32, 5-6 seedlings per tube) and different letters denote statistically significant differences by ANOVA ($P<0.05$, 340 degrees of freedom, LSD 5%) from log transformed data.

Appendix V. Real-time PCR analysis of expression of genes related to N signalling under various N treatments and in the presence of CPT (non-transformed data)

12.1 Results

Real-time PCR analysis of expression of genes related to N signalling under various N treatments and in the presence of CPT is presented graphically using non-transformed mean NRQ values. Different letters denote statistically significant differences identified by ANOVA (p<0.05, 20 degrees of freedom, LSD 5%) on $Log_2(1/NRQ)$ log transformed data.
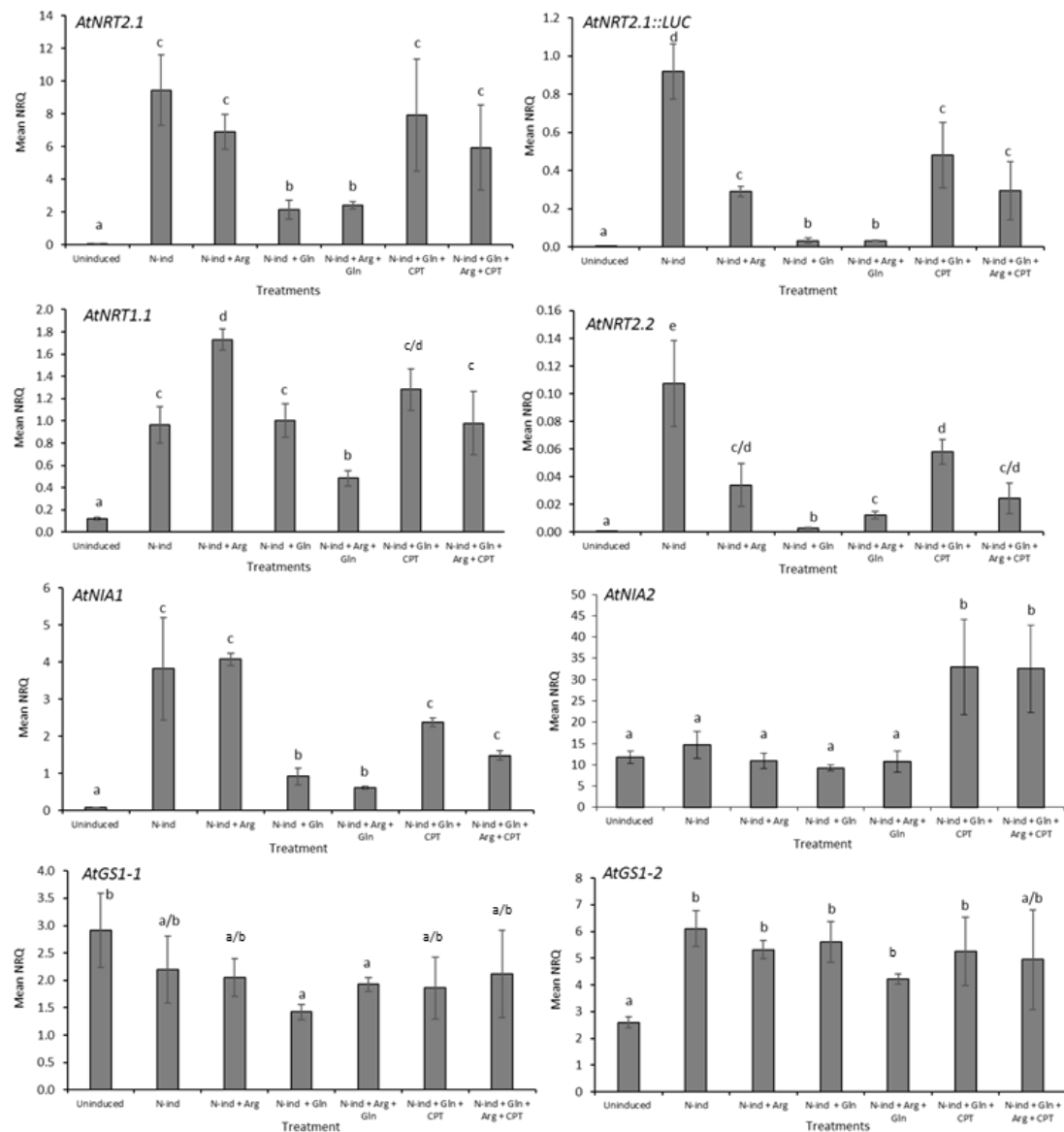
Figure 12.1 Real-time PCR analysis of expression of genes related to N signalling under various N treatments and in the presence of CPT *pNRT2.1::LUC* seedlings were cultured using the FrameStrip deep-well method with a background concentration of 100 µM NH_4NO_3. Where used, CPT was applied at 100 µM to the wells, to 12 day old seedlings and N treatments were applied a day later. Treatments were: N-ind (induced, 0.3 mM KNO_3); N-ind + Arg (0.3 mM KNO_3 + 4 mM Arg); N-ind + Gln (0.3 mM KNO_3 + 10 mM Gln); N-ind + Arg + Gln (0.3 mM KNO_3 + 4 mM Arg + 10 mM Gln); N-ind + Gln + CPT (0.3 mM KNO_3 + 10 mM Gln + 100 µM CPT); N-ind + Gln + Arg + CPT (0.3 mM KNO_3 + 4 mM Arg + 10 mM Gln + 100 µM CPT). Mean Normalised Relative Quantification (NRQ) values shown ±SE (n= 3 independent replicates, root material for each was from a separate deep-well plate), non-transformed data are shown. Different letters denote statistically significant differences identified by ANOVA (p<0.05, 20 degrees of freedom, LSD 5%) on Log_2(1/NRQ) log transformed data.

Appendix VI. Using root growth of topoisomerase I Arabidopsis mutants as a proxy for CPT inhibition sensitivity (non-transformed data)
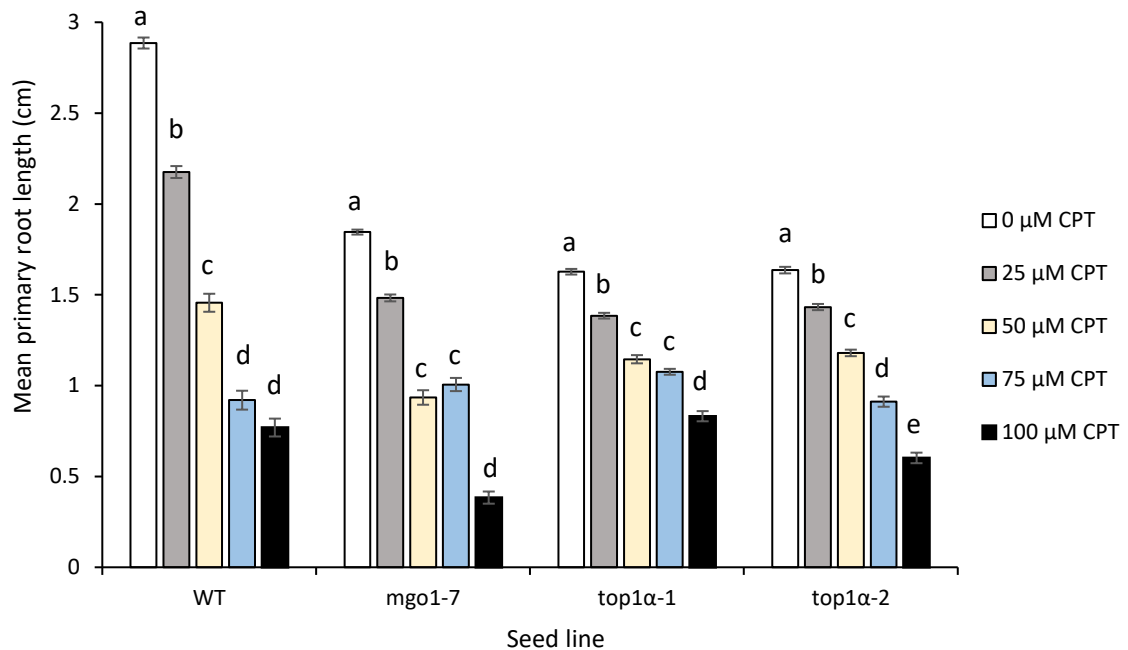

Figure 13.1 Using root growth of topoisomerase I Arabidopsis mutants as a proxy for CPT inhibition sensitivity Seedlings of wild-type and three topoisomerase I mutants (mgo1-7, top1α-1, top1α-2) were grown for 5 days on vertical agar plates (Section 2.5). Eight seedlings were selected for uniformity of primary root growth and transferred to new vertical agar plates containing 1 mM KNO_3 and CPT at the specified concentrations. The increase in primary root length over the following 7 days was measured. Values are means of primary root length (cm) ±SE (n = 8 individual seedlings). Different letters denote statistically significant differences identified within each seed line by ANOVA ($P<0.05$, 157 degrees of freedom, LSD 5%) for log transformed data.

Appendix VII. Investigating conditions that elicit N-repression using high nitrogen insensitive (*hni*) mutants

14.1 Results

Girin et al. (2010) previously used the *pNRT2.1::LUC* reporter line in a screen to identify mutants that displayed enhanced luminescence in the presence of 10 mM NH_4NO_3. Two of these high nitrogen insensitive mutants (*hni140-1* and *hni48-1*) have been used to determine whether they are also insensitive to the conditions of N-repression that have been used in the present study. (Unfortunately seed of a third line, *hni9-1,* was found to be non-viable and so it could not be included in this experiment). Seedlings were grown in the FrameStrip platform for 13 days and then the roots treated with 0.3 mM nitrate + 4 mM Arg to induce *pNRT2.1::LUC* expression. One batch of nitrate-induced seedlings was treated in addition with 10 mM Gln and another batch with 10 mM NH_4NO_3. The results in Fig. 8.1 show that nitrate induction occurred in all three lines, as expected, although much more strongly in *hni48-1* than in the wild-type. However, the NH_4NO_3 treatment strongly repressed *pNRT2.1::LUC* expression in both mutant lines, such that luminescence was not significantly different from the wild-type. Similarly, the 10 mM Gln treatment was equally or more effective in repressing expression of the *pNRT2.1::LUC* gene in the *hni* mutants than in the wild-type. Unexpectedly, the use of 10 mM NH_4NO_4 significantly repressed luminescence in both *hni* mutants, to a similar degree as in the wild-type background
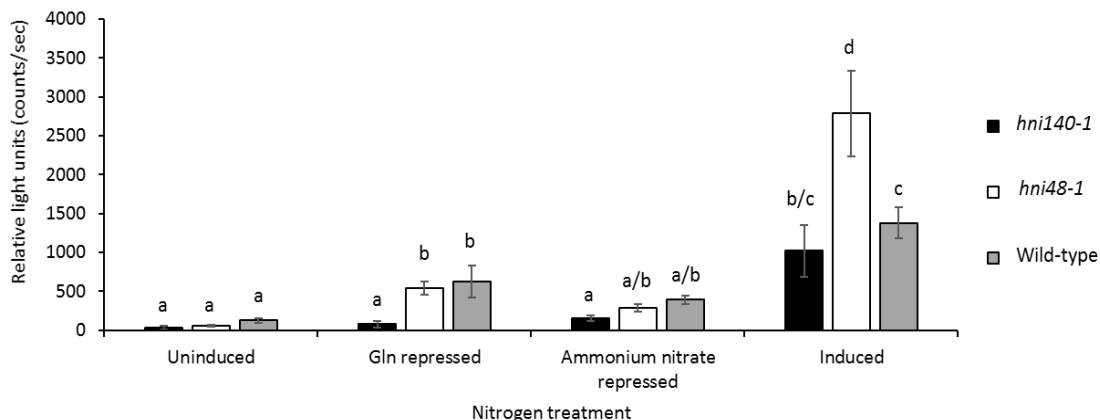
Figure 14.1 Investigating the effect of different N-repression conditions on two *hni* mutants *pNRT2.1::LUC* and *hni* mutant lines (*hni*140-1, *hni*48-1) were cultured using the luciferase screening platform containing 100 μM NH₄NO₃. N treatments were applied to 13 day old seedlings and conditions were: uninduced, (background N only); Gln-repressed (0.3 mM KNO₃ + 4 mM Arg + 10 mM Gln; Ammonium nitrate repressed (10 mM NH₄NO₃ + 4 mM Arg); Induced (0.3 mM KNO₃ + 4 mM Arg). Luciferase assay performed in SpectraMaxi3 luminometer (Section 2.7.7). Values are means ±SE (16 individual seedlings). Different letters denote statistically significant differences identified by ANOVA (P<0.05, 192 degrees of freedom, LSD 5%).

14.2 Discussion

14.2.1 Investigating different conditions that elicit N-repression using *hni* Arabidopsis mutants

Given that the *hni* mutants have been shown to be insensitive to high ammonium nitrate conditions (Girin et al., 2010) their response to N-repression using Gln in the presence of nitrate was investigated. Fig. 8.1 shows that *pNRT2.1::LUC* expression in both the *hni140-1* and *hni48-1* mutants was significantly repressed by Gln-repression conditions. However unexpectedly they were also repressed under the ammonium nitrate concentrations used by Girin et al. (2010). Both studies also used the same nitrate induction concentrations, yet *hni48-1* was strongly stimulated above the *pNRT2.1::LUC* and *hni140-1* lines only here. A hypothesis to explain the altered luciferase expression in Fig. 6.4 is that Arg,

given its repressive effect on N signalling genes in Fig. 6.3, contributed to the repression of luciferase expression in these lines when added with ammonium nitrate. This could also explain the strongly stimulated response of luminescence in the induced *hni48-1* mutant in the presence of Arg. Repeating this experiment without Arg could provide useful information to determine if Gln-repression is elicited in the same manner in the mutants and determine if Arg, in the presence of ammonium nitrate alters their response to high N. Girin et al. (2010) using the same *hni* mutants in this research reported that *NRT2.1* expression insensitive to high N conditions (10 mM NH_4NO_3) and when placed in low nitrate induction conditions luminescence was indistinguishable from the wild-type. They used an additional mutant line, *hni9-1*, which showed the greatest insensitivity to high N-repression, while this seed line was sourced for investigation here it was not included due to seed viability issues.